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GALACTOSEMIA, A CONGENITAL DEFECT IN A NUCLEOTIDE TRANSFERASE: A PRELIMINARY REPORT

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Communicated by Gerty T. Cori, December 22 1955.

Galactosemia is a disease of childhood which manifests itself biochemically as a disorder in the metabolism of galactose (cf. Hartmann[1]) as well as of α-galactose-1-phosphate (Gal-1-P).[2] The latter ester has recently been shown to accumulate in the blood of galactosemic infants after the administration of galactose.[2]

According to Leloir,[3] the conversion of Gal-1-P to α-glucose-1-phosphate (G-1-P) involves the following steps, in which a nucleotide, uridinediphospho-glucose (UDPG) is required:

$$\text{Gal-1-P} + \text{UDPG} \rightleftharpoons \text{G-1-P} + \text{UDPGal}, \quad (1)$$

$$\text{UDPGal} \rightleftharpoons \text{UDPG}. \quad (2)$$

Step (2) is catalyzed by an enzyme discovered by Leloir[4] in 1951 and called "galacto-waldenase." Step (1) was found to be catalyzed by an enzyme which has been found in galactose-adapted yeast[5] and mammalian liver.[6] We call this enzyme "PGal-transferase." It has been postulated at various times that galactosemia is a defect due to a block in the synthesis of galacto-waldenase. The fact that Gal-1-P accumulates in the erythrocytes of galactosemic subjects when galactose is administered, together with the fact that the clinical symptoms completely disappear if the patients are put on a galactose-free diet, would be compatible with a block in step (1), i.e., PGal-transferase, rather than with a block in step (2) catalyzed by galacto-waldenase.

Methods and Materials.—Hemolyzates were used as the enzyme source. The incubation time was 30 minutes. One sample was incubated with Gal-1-P alone and the second with UDPG alone. The missing substrate was added after deproteinization. The third sample was incubated with both substrates. For the determination of Gal-1-P, UDPG, and UDPGal specific enzymatic methods were used. The indicator in these methods is one of the pyridine nucleotides (TPN or DPN), both of which upon reduction develop an increase in absorption at 340 mμ. The Gal-1-P was determined by means of its liberation of G-1-P from UDPG, using purified PGal transferase and a TPN indicator system.[7]

UDPG was determined by means of a specific, purified UDPG dehydrogenase,[8] using DPN as indicator. UDPGal was determined by the same principle, except that galacto-waldenase (fractionated from liver[6]) was also added.[9] Alpha Gal-1-P was kindly made available to us through Drs. Hewitt G. Fletcher, Jr., and Elizabeth Maxwell. UDPG was a commercial product from the Sigma Chemical Company, St. Louis, Missouri. UDPGal was prepared by enzymatic techniques.[6]

Results.—We have confirmed and extended the observation of Schwartz *et al.*[2] that galactose added to erythrocytes from galactosemic children gives rise to accumulation of Gal-1-P. The erythrocytes were incubated in vitro with galactose for three hours at 37° and the Gal-1-P determined enzymatically.[7] Blood from normal children showed no accumulation of Gal-1-P under these conditions (less than 0.01 μM per milliliter of red blood cells per hour), whereas, in three cases of galactosemia, about 0.1 μM of Gal-1-P accumulated per milliliter of red blood cells per hour.

The presence of PGal transferase was studied by means of incubating Gal-1-P and UDPG together with hemolyzates and subsequently measuring by enzymatic techniques the conversion of UDPG to UDPGal.[8, 9]

In order to insure that the hemolyzates were not fortuitously varying in activity with respect to this class of enzymes, another nucleotide transferase, which we call "PP transferase,"[10] was also measured by the same type of method and at the same time as the PGal transferase. The PP transferase catalyzes the following reaction, which involves inorganic pyrophosphate (PP) and uridine triphosphate (UTP): G-1-P + UTP $\rightleftharpoons$ PP + UDPG. The PP transferase does not play a direct role in the metabolism of Gal-1-P.[7] It brings about a conversion of G-1-P to UDPG, which by subsequent enzymatic dehydrogenation is converted to UDP-glucuronic acid,[11] or, alternatively, without oxidation can be converted into UDPGal by the above-mentioned enzyme, galacto-waldenase.[4]

In Table 1, average values of PGal and PP transferases for three groups of

TABLE 1

AVERAGE RATES FOR PGAL AND PP TRANSFERASES IN HUMAN HEMOLYZATES FROM NONGALACTOSEMIC AND GALACTOSEMIC SUBJECTS

No. of Cases	Types of Cases	Diet	μM UDPG Exchanged per Milliliter Red Blood Cells per Hour	
			PGal Transf.	PP Transf.
11	Normal	Ordinary	0.75	1.10
3	Milk allergies	Galactose-free	0.88	1.35
8	Galactosemic	Galactose-free	<0.02	1.85

subjects are presented. The first group comprises normal subjects of various ages (male and female); the second group deals with individuals on galactose-free diets on account of milk allergy (three cases); and the third group deals with galactosemic subjects (eight cases). It can be seen that blood from the galactosemic subjects is devoid of PGal transferase (less than 3 per cent, which means essentially undetectable). The PP transferase, however, is constantly present not only in the group of normal and milk-allergy subjects but also in the galactosemic subjects.

We therefore propose that galactosemia is an inborn defect, presumably of genetic origin,[12] in the production of PGal transferase.

Summary.—Galactosemia seems to furnish an example of a congenital human metabolic disease in which a specific enzyme is missing. The enzyme which

catalyzes the exchange of α-galactose-1-phosphate with uridinediphospho-glucose, forming α-glucose-1-phosphate and uridinediphospho-galactose, is absent in blood from galactosemic subjects. It is known that this enzymatic exchange is an important step reaction by which administered galactose is used in general carbohydrate metabolism. Several of the metabolic manifestations of the disease might readily be explained on the basis of this enzymatic defect.

Our thanks are due to Drs. G. T. Cori, V. O'Donnell, J. Kety, H. H. Mason, R. Harris, and A. Hartmann for their kind help in obtaining the galactosemic cases, and to Miss Bodil Waage-Jensen, who rendered valuable assistance as a trainee under the Scandinavian-American Foundation through a grant-in-aid generously made available to one of us (H. M. K.) by the Eli Lilly Laboratories.

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THE COMBINING POWER OF MYOGLOBIN FOR ALKYL ISOCYANIDES AND THE STRUCTURE OF THE MYOGLOBIN MOLECULE

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Communicated November 26, 1955

It was found by St. George and Pauling[1] that, although the combining powers of ferroheme with ethyl isocyanide, isopropyl isocyanide, and tertiary butyl isocyanide are essentially the same (the extreme values of the equilibrium constants differing by only a factor of 3), hemoglobin combines far more strongly with ethyl isocyanide than with *t*-butyl isocyanide, corresponding to a factor of 200 in the equilibrium constants, with isopropyl isocyanide having an intermediate value. These facts led St. George and Pauling to the conclusion that there is steric hindrance between the alkyl groups of the isocyanides and a part of the globin in the hemoglobin molecule. Inasmuch as it is known that the heme group is attached to globin by

way of an imidazole ring of a histidine side chain of the globin on the side opposite that to which the alkyl isocyanide molecule is attached, it was concluded that the heme groups of hemoglobin are buried within the protein molecule.

The rather close similarity in properties of myoglobin and hemoglobin suggests a general similarity in structure. The myoglobin molecule, with molecular weight 17,000, is, however, only one-quarter as large as the hemoglobin molecule, and it is conceivable that the myoglobin molecule might differ from the hemoglobin molecule in having the heme attached on the surface of the globin of myoglobin; it would then be predicted that myoglobin would have nearly the same combining powers for alkyl isocyanides differing in size and shape of the alkyl group, whereas if the heme were buried in the globin the combining powers would be different, as in the case of hemoglobin.

We have determined equilibrium constants for combination of horse myoglobin with ethyl isocyanide, *n*-propyl isocyanide, isopropyl isocyanide, and *t*-butyl isocyanide and have obtained the values 2.0×10^5, 1.1×10^5, 1.4×10^4, and 1.0×10^3, respectively. The value for ethyl isocyanide is accordingly 200 times greater than that for *t*-butyl isocyanide. This great dependence of the value of the combining constant on the nature of the alkyl group has a reasonable explanation in steric hindrance, and we conclude that in myoglobin, as in hemoglobin, the heme group is not attached to a surface of the globin molecule, but is buried within it.

Experimental Procedure and Results.—Myoglobin was prepared from horse heart according to a method adapted from Theorell[2] and Morgan.[3] A 4.5-kg. heart, obtained from the abattoir about 12 hours prior to use and kept refrigerated during this time, was washed, dissected free of fat and connective tissue, and cut into small pieces. Weighed portions together with half their weight in water were triturated in a Waring Blendor for between 30 and 45 seconds. This mixture was refrigerated for about 15 hours, and the fluid was removed by filtration, yielding 2.1 l. of extract with pH 5.8. The extract was neutralized with 1 *N* NaOH, and 540 ml. of 1 *F* basic lead acetate solution[4] was added slowly with stirring. The precipitate that formed was removed by centrifugation, and the supernatant fluid was brought to pH 6.6 and made 3 *F* in phosphate by adding a suitable mixture of KH_2PO_4 and K_2HPO_4;[5] this amount of phosphate saturated the solution at 4° C. The resulting precipitate, containing excess lead (as phosphate) as well as hemoglobin,[3] was removed by centrifugation, and the supernatant solution was dialyzed against several changes of saturated ammonium sulfate. The precipitated protein was removed by filtration, washed with saturated ammonium sulfate, dissolved in a minimum volume of water, and then again dialyzed against saturated ammonium sulfate. Microscopic examination of the resulting precipitate disclosed that, although some amorphous material was present, fanlike clusters of needles predominated. The precipitate was dissolved in a minimum volume of water, and the solution was dialyzed against 0.133 *F* KH_2PO_4-Na_2HPO_4 at pH 6.8. Spectrophotometric examination of this solution disclosed maxima and minima in close agreement with those given by Theorell[6] for ferrimyoglobin; following reduction with sodium dithionite, the absorption spectrum for ferromyoglobin was found; and after treatment with carbon monoxide the distinctive absorption spectrum of carbonmonoxymyoglobin was revealed. Electrophoretic examination in barbital buffer, ionic strength 0.1 and pH 8.6, disclosed essentially a single component

(mobility, 1.95×10^{-5} cm^2/volt sec), with the exception of a small contaminant having a negative refractive index contribution, possibly a lipoprotein, which moved off rapidly (mobility, 1.81×10^{-4} cm^2/volt sec) toward the anode. Concentrations of myoglobin solutions determined spectrophotometrically, using extinction coefficients given by Theorell,[6] agreed very closely with those determined by differential refractometry.

Alkyl isocyanides were prepared from alkyl iodides by the silver cyanide method,[7] using two moles of silver cyanide per mole of alkyl iodide, instead of equimolar quantities as stipulated in the published method. (The authors are indebted to Dr. Jake Bello for synthesizing the isocyanides used in this work.) Binding curves were obtained at 25° C. by adding serial increments of the solutions of isocyanides to the myoglobin, which had first been reduced from the ferrimyoglobin to the ferromyoglobin form with sodium dithionite. Three milliliters of 0.16 per cent myoglobin solution was placed in a suitable cuvette, and solutions of the isocyanides were made of such concentration that a maximum of 32 μl. of isocyanide solution (delivered from an ultramicroburette) was required to saturate the myoglobin.

With the addition of the isocyanides to the myoglobin, absorption maxima appeared at 532 and about 563 mμ. Since the change in optical density with increments of isocyanides was greater at 532 than at 563 mμ, measurements at 532 mμ were used to determine the amount of binding of isocyanide by the myoglobin. Percentage saturation was calculated from the ratio of the change observed to the total change in optical density at saturation. Assuming a molecular weight of 17,000 for myoglobin and the binding of one mole of isocyanide per mole of myoglobin at saturation, the amount of isocyanide bound and the concentration of free isocyanide were calculated for each addition of this material.

As a preliminary to the isocyanide-binding study, the spectral characteristics of myoglobin and some of its derivatives were studied in the visible range from 475 to 675 mμ. Table 1 summarizes the absorption maxima found in this range. As

TABLE 1

Compound	Absorption Maxima (Mμ)	Compound	Absorption Maximum (Mμ)
Ferrimyoglobin	502, 631	*n*-Propyl isocyanide–myoglobin	532, 563
Ferromyoglobin	556	Isopropyl isocyanide–myoglobin	532, 563
Carbonmonoxymyoglobin	542, 579	*t*-Butyl isocyanide–myoglobin	532, 562
Ethyl isocyanide–myoglobin	532, 566		

indicated in the table, the β band at 532 mμ was not influenced by the size of the alkyl isocyanide; however, the α band, at 566 mμ for the ethyl isocyanide–myoglobin complex, shifted slightly toward the blue with increasing size of the isocyanide alkyl group.

Using the symbols "M" for myoglobin, "I" for isocyanide, and "MI" for myoglobin-isocyanide, the equilibrium constant K for the combination of myoglobin and isocyanide is equal to [MI]/[M][I]. For each of the four isocyanides, values of the equilibrium constant were calculated for each of a half-dozen experimental determinations in the range 20 to 84 per cent saturation of the myoglobin. In order to eliminate a trend in the values of K obtained for ethyl isocyanide, a 2 per cent correction in the concentration of the stock solution was made; a similar correction

was also made for *n*-propyl isocyanide, which was suspected, because of its color, to be contaminated with a small amount of *n*-propyl iodide. The values of the equilibrium constants were averaged, with the concentration of free isocyanide as a weight factor. The equilibrium constants obtained in this way are 2.0×10^5 for ethyl isocyanide, 1.1×10^5 for *n*-propyl isocyanide, 1.4×10^4 for isopropyl isocyanide, and 1.0×10^3 for *t*-butyl isocyanide, the units in each case being liters per mole.

* Contribution No. 2059.

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THE CONTROL OF A CANCER GROWTH IN EMBRYONATED EGGS

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Most of the investigations in our laboratories having to do directly with cancer and cancer tissues in the last ten years have centered around the development by one of us[1, 2] of a convenient method for inoculating and propagating cancer tissue in the yolk sac of embryonated eggs. This method has been developed to a point such that it is now possible to inoculate a series of eggs quantitatively and to harvest several days later a relatively uniform series of egg-grown cancers, along with chick embryos which have grown alongside, utilizing the same blood supply. Each fertile egg will bear a tumor, and without dealing with large numbers it is generally possible to get satisfactory replicate results from different sets of eggs.

Although there is the expected variation in the sizes of tumors harvested at any given time, this variation is not prohibitively large and does not include zero values. The following results may be regarded as typical. When eggs were inoculated with a suitable tumor suspension on the fourth day of incubation, the tumor weights, expressed in grams, at the end of the tenth day were 0.19, 0.12, 0.17, 0.08, 0.05, 0.09, and 0.04. At the end of thirteen days, in a similar set, the respective tumor weights in grams were as follows: 0.6, 0.9, 0.6, 0.4, 0.3, 0.5, 0.6, and 1.0.

This relative uniformity and consistency of growth make it possible to subject various sets of eggs to different treatments and, by comparison with controls, to determine the effects of different treatments both on the cancer growth and on the growth of the chick embryos. Many thousands of experiments involving hundreds of thousands of eggs have been carried out in our laboratories using this technique.

The location of the tumor in the yolk sac is associated with minimum interference with the supporting chick embryo. The chick membranes remain in their normal condition, and compounds being tested for possible anticancer activity can be introduced into the tumor-bearing egg over the membrane blood vessels, analogous to subdermal injection in a free-living animal.[3] The embryo serves as a control of rapidly growing nontumor tissue.

It has been noted, both in our laboratories and elsewhere, that serial transplantation of mouse or rat tumors in eggs has adverse effects on individual chick embryos.[4] This, if not controlled, will lead to irregular tumor size in the eggs of a particular experiment. There is much variation in the way the individual eggs of a group receiving the same tumor suspension react to the toxic tendency. We have eliminated this difficulty by keeping a tumor line going in the host animal. Serial transplants of a tumor in eggs used for our chemotherapy tests are not continued beyond the third generation before resorting again to mouse-grown tumor tissue. With this procedure the tumors are more uniform and grow better, and the tumor-bearing eggs have a higher survival rate.

This tendency for serial transplants of tumor tissue in the egg to become irregularly toxic to the host embryo may have fundamental significance. The phenomenon is very striking in association with certain tumors. In some instances, even after two or three serial egg transplants of a tumor, toxic effects are manifested by an increasing mortality rate in the inoculated eggs. We have cultivated the RC mouse mammary tumor by continuous egg-to-egg transplantation for over ten years. The embryos of many of the eggs are severely affected. The series has been maintained by allowing the tumor-bearing eggs to continue to about the seventeenth day of incubation and then selecting tumor tissue for further transplants from the eggs which do not manifest the toxic effect. Usually, by the seventeenth day of incubation nearly all the affected embryos are dead. Without such selection the mortality in the inoculated eggs rises rapidly and would soon make it impossible to continue the egg propagation of this tumor.

In our opinion this transfer toxic effect may very well indicate the presence in these tumors of a virus-like tumor agent which changes or mutates in association with the alien egg environment. It is well known that viruses tend to mutate when cultivated under changed conditions. It is noteworthy that the adverse effects observed when a mouse or rat tumor is cultivated continuously in embryonated eggs also extend to the tumor itself. Under such circumstances the tumor growth is inhibited, and its gross appearance is changed.

Much of our egg work for the past several years has been concerned with the extensive testing of various compounds and extracts for their possible anticancer properties. A C3H mouse mammary carcinoma has been the source tumor for the egg inoculations.[2] This tumor grows rapidly in eggs, about doubling its size in the 24-hour period from the eleventh to the twelfth day of incubation.

We have been mindful of the limitations as well as of the advantages of this method of study. Much of our investigation has involved a particular tumor. Although our own work on the quantitative distribution of B vitamins in cancers,[5] as well as that of others on enzyme content,[6, 7] has indicated that tumors induced in different ways and from different sources have much in common, we are fully aware that a specific treatment capable of giving certain results with one tumor may not give duplicate results with another tumor.[7, 8]

We are also aware that a specific tumor cultivated in embryonated eggs cannot be expected to behave identically with the same tumor grown in its normal host rat or mouse. It is also obvious that the means which might be used to control a cancerous growth in mice or rats could not be carried over unchanged for cancer control in human beings.

Notwithstanding these limitations, the relatively cheaply and easily applied technique that we now have available provides an excellent tool for long-range investigation in cancer control. It would seem that if we are not able to solve the problem of cancer control in eggs, we are less likely to be successful with the more complex system inherent in experimental animals. Although no one can foresee the pathway by which cancer investigation will progress, it seems likely that it may take a course involving, first, the control of cancer in embryonated eggs; second, its control in experimental animals; and, last, its control in human patients.

Our investigations have now reached a point at which we are able to control with substantial success the growth of a particular mouse mammary tumor when it is cultivated in embryonated eggs. In a series of cancer-inoculated eggs 57 out of 58 control embryos had been killed by the sixteenth day of incubation by tumor growth. The remaining one bore a large tumor. In a parallel series of 140 cancer-inoculated eggs in which treatment (to be described later) was applied, 86 embryos remained alive on the sixteenth day. Among these, 81 contained mere traces of what may have been viable cancer tissue, but in 5 there were no observable traces. The effects of the cancer inoculation have thus been largely counteracted by the treatment, and we believe that this result merits careful attention. This is particularly true because the treatment used has allowed the chick embryo, sharing one blood stream with the tumor, to grow in a normal manner. We recognize that these results constitute only a beginning. After many years of testing numerous compounds, with dubious results, including many of those which had given some promise in other laboratories, we are encouraged by the progress made.

One of the difficulties in getting able men to work in the cancer field centers is the fact that the ultimate goal seems too far off and progress is not easy to evaluate. Our gleam of success in dealing with a particular cancer-host system (tumor-embryonated eggs) which we have selected for study gives us a concrete reason for encouragement.

A number of different agents have been found in various laboratories to be unfavorable to the growth of certain tumors. In most, if not all, instances, the effective compounds are very toxic also to nontumor tissue.[8] Our success in controlling an egg-cultivated tumor lies in the simultaneous use of more than one unfavorable influence. Several investigators have tried combinations of different chemical compounds in an effort to discover substances which would act synergistically against tumor growths.[9] In our work we have used this approach but have extended it to include not only synergistic chemicals but also the combined effect of chemical and physical agents. Our best success to date has resulted from the combined use of compounds capable of inhibiting tumor growth and a moderate increase in temperature which has previously been found to be, by itself, unfavorable to cancer growth.[10]

We have found that the effectiveness of a chemical compound which inhibits tumor growth is increased by raising the temperature of tumor-bearing eggs from the normal 37.2° to 40° C. for 24–48 hours. For example, aminopterin (4-aminopteroylglutamic acid), which is known to inhibit the growth of some tumors and is also exceedingly toxic to certain nontumor tissues, when injected at a relatively nontoxic dosage over the embryo membrane blood vessels of tumor-bearing eggs incubated for a 24-hour period at the normal 37.2° temperature, inhibited growth of the tumors to the extent that they were 62 per cent as large as those in the control eggs injected with the suspending fluid. At the same time the supporting chick embryos of the experimental eggs were 80 per cent as large as the control embryos. Eggs from this same experiment which were incubated for 24 hours at 40° contained tumors with zero growth (actually a decrease in size) as compared to controls subjected to the same temperature, and the embryos were 102 per cent the size of control embryos. Thus it appears that in association with a raised temperature the aminopterin was more effective against the tumor and less toxic to the embryo (Table 1).

TABLE 1

EFFECT OF AMINOPTERIN ON GROWTH OF EGG-CULTIVATED MOUSE MAMMARY CANCER AT 37.2° AND AT 40° C.
(Duration of Test: 24 Hours [12th–13th Day of Incubation]; 0.02 Mg. Injected, as Oil-H_2O Suspension, over Embryonic Membranes)

TEMPERATURE (° C.)	NO. OF EXPS.	TOTAL NO. OF EGGS	NO. OF EGGS SURVIVING	SURVIVAL (CONTROL = 100)	AV. INITIAL TUMOR SIZE (GM.)	AV. TERMINAL TUMOR SIZE (GM.)	AV. TERMINAL EMBRYO SIZE (GM.)	GROWTH FOR PERIOD OF TEST (CONTROL = 100)	
								Tumor	Embryo
Control, 37.2	4	65	52	100	0.31 ± 0.03	0.52 ± 0.04	5.30	100	100
Expt., 37.2	4	39	31	99	.31 ± .03	.44 ± .07	5.06	62	80
Control, 40	4	65	49	100	.31 ± .03	.49 ± .09	5.47	100	100
Expt., 40	4	39	31	105	0.31 ± 0.03	0.23 ± 0.02	5.48	0	102

Triethylenemelamine (TEM), which has received much attention for its tumor-inhibiting properties,[8] plus increased temperature was our most effective combination. The C3H mouse mammary tumor used in these experiments is susceptible to this compound. Two types of tests were carried out. The first involved the injection of the experimental eggs over the embryonic membranes with TEM on the tenth day of incubation, and the controls, as usual, were injected with the suspending fluid (water). Half the controls and half the experimentals were incubated at the regular 37.2° C. temperature, and the remaining eggs were incubated at 40°. On the twelfth day of incubation, or after a lapse of 48 hours, the 37.2° experimentals contained tumors which averaged 19 per cent of the control tumor weight, while the tumors from the 40° experimental group showed no growth or some regression (Table 2). There was no exception to this tendency in any one of the seven experiments on which Table 2 is based.

A further series of five experiments was carried out with the objective of determining whether the effect noted in Tables 1 and 2 would continue if the eggs with only the one injection were allowed to continue their incubation for a longer period (Table 3). Tumor-bearing eggs were injected over the embryonic membranes with TEM on the tenth day of incubation, and controls were prepared as in the previous experiments. Half the eggs were incubated at 37.2° and half at 40° C.

for 48 hours. At the end of this period the eggs incubated at 40° were put back into the 37.2° incubators. Each of these experiments was terminated on the sixteenth day of incubation. At this time there were 5 control eggs alive from the total of 56 used in the 5 experiments of the 37.2° incubated group, and only 1 control egg remained alive from the 40°–37.2° group. On the other hand, 96 of the 136 eggs of the experimental 37.2° group and 86 of the 140 eggs of the experimental 40°–37.2° group survived. When the eggs were autopsied, it was found

TABLE 2

EFFECT OF TRIETHYLENEMELAMINE ON GROWTH OF EGG-CULTIVATED MOUSE CANCER AT 37.2° AND AT 40° C.
(Duration of Test: 48 Hours [10th–12th Day of Incubation]; 0.2 Mg. Injected over Embryonic Membranes)

TEMPERATURE (° C.)	NO. OF EXPS.	TOTAL NO. OF EGGS	NO. OF EGGS SURVIVING	SURVIVAL (CONTROL = 100)	AV. INITIAL TUMOR SIZE (GM.)	AV. TERMINAL TUMOR SIZE (GM.)	AV. TERMINAL EMBRYO SIZE (GM.)	GROWTH FOR PERIOD OF TEST (CONTROL = 100) TUMOR	GROWTH FOR PERIOD OF TEST (CONTROL = 100) EMBRYO
Control, 37.2	7	79	63	100	0.07 ± 0.02	0.28 ± 0.06	3.66	100	100
Expt., 37.2	7	77	53	86	.07 ± .02	.11 ± .03	3.61	19	96
Control, 40	7	81	44	100	.07 ± .02	.31 ± .06	4.08	100	100
Expt., 40	7	81	51	117	0.07 ± 0.02	0.06 ± 0.02	4.04	0	98

that the group treated at 37.2° contained normal-appearing tumors which averaged in size 25 per cent of those contained in the corresponding untreated controls. The control tumor size was based on the average weight of the few remaining alive (associated with live embryos) plus the tumors from the embryos which died before the termination of the experiment. The 40°–37.2° experimental group contained tumors which averaged only 9 per cent of those of the corresponding controls. Further, the slight amount of tumor tissue present in the eggs kept at

TABLE 3

INFLUENCE OF TEMPERATURE ON REACTION OF EGG-CULTIVATED MOUSE MAMMARY TUMOR TO TRIETHYLENEMELAMINE
(Duration of Test: 6 Days [10th–16th Day of Incubation]; Incubation Temperatures: (1) 37.2° C., (2) 40° C. for 2 days and 37.2° C. for 4 days; 0.2 Mg. Injected over Embryonic Membranes)

TEMPERATURE (° C.)	NO. OF EXPS.	TOTAL NO. OF EGGS	NO. OF EGGS SURVIVING	AV. TUMOR SIZE (GM.)	EMBRYO SIZE (GM.)	TUMOR SIZE (CONTROL = 100)	NO. OF EGGS WITHOUT TUMOR
(1)							
Control, 37.2	5	56	5	1.33	...	100	0
Expt., 37.2	5	136	96	0.33 ± 0.14	7.92	25	2
(2)							
Control, 40, 37.2	5	57	1	1.39	...	100	0
Expt., 40, 37.2	5	140	86	0.13 ± 0.05	9.24	9	5

higher temperature was generally atypical in appearance, indicating doubtful viability. It will also be noted in Table 3 that the embryos of the 37.2° experimental group averaged 7.92 gm., whereas the average weight of the embryos of the higher-temperature experimental eggs was 9.24 gm.

We have no wish to overemphasize the implications of our experiments for cancer therapy. But in a field where any kind of positive result is rare, it does seem that this work warrants some attention—not so much because of any practical accomplishment but rather because of the approach, which could lead to further

progress and the establishment of some general principles which apply to cancer control. The yolk sac tumor-bearing egg provides a relatively accessible tumor-nontumor system, and one which can easily be subjected to a variety of manipulations. In addition to the introduction of such variables as chemicals and temperature changes, others, such as X-rays and other types of radiation, can readily be utilized either singly or in combination.

There is a possibility or even a probability that successful cancer treatment will ultimately involve the simultaneous use of more than one type of agent. It may require suitable drugs along with suitable physical agents. Our use of the egg embryo–cancer system has given indication that this is so and should be useful in the further exploration of this possibility.

Summary.—It is possible by inoculating tumors into the yolk sac of embryonated eggs to obtain consistent and relatively uniform cancer growths in every fertile egg and to harvest these growths along with the chick embryos which have grown alongside, utilizing the same blood stream. With the tumor attached to the inner lining of the yolk sac, test compounds can be injected over the blood vessels of the chick membranes, analogous to subdermal injection.

Using this type of tumor-embryo system, it was discovered that a rise in temperature from the usual incubation temperature of 37.2°–40° C. for a period of 24–48 hours increased the anticancer effects of such compounds as aminopterin and triethylenemelamine. In experiments with TEM plus raised temperature for 48 hours, in which the tumor-bearing eggs received one injection of the compound at the tenth day of incubation, 86 of the group of 140 eggs survived to the sixteenth day of incubation, at which time only traces of atypically appearing tumor were present in most of them. Of 57 controls in the same experiment, all but one were killed by the growing cancers; one survived to the sixteenth day and contained a large tumor.

We suggest that cancer control may be found to involve the simultaneous use of chemical and physical agents. The egg embryo–cancer system can be used more readily to subject this possibility to laboratory tests.

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*HYDROGEN-BONDED HELICAL CONFIGURATIONS OF POLYNUCLEOTIDES**

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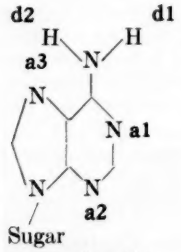
Communicated by L. Pauling, November 23, 1955

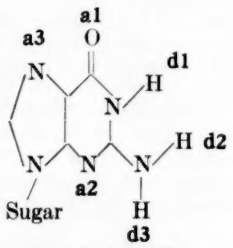
The structure for the sodium salt of deoxyribonucleic acid (DNA) proposed by Watson and Crick[1] requires, in agreement with the more recent analytical data, the occurrence in the molecule of equimolar amounts of adenine and thymine, on the one hand, and of guanine and cytosine, on the other. Since it is by no means certain that all nucleic acids, whether DNA or ribonucleic acid, whatever the source, will conform to this composition, it becomes of interest to inquire into the possibility of other structures which satisfy the chemical evidence concerning the bonding in a single nucleotide chain and which also conform to our knowledge of molecular structures. The basic feature of the Watson-Crick structure is the arrangement of the four bases: these come in the pairs, above, connected by hydrogen bonds which link two polynucleotide chains together, and lie perpendicular to the helical axis of the molecule, 3.4 A apart.

A systematic study of structures which also have the bases perpendicular to a helical axis is described below. Although it may be possible to construct a polynucleotide molecule consisting of a single chain internally linked with hydrogen bonds, it seems reasonable to retain the planar separation of the basis, necessitating two or more nucleotide chains winding about a common helical axis.

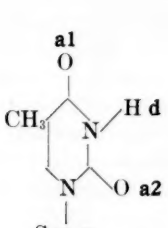
We first consider base-base pairs, and, in order to facilitate their description, we number the hydrogen-bonding donor and acceptor sites as follows:



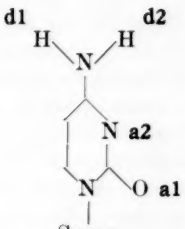
Adenine (A)



Guanine (G)



Thymine (T)



Cytosine (C)

Precise X-ray crystallographic information on these and related compounds is meager. The models used had rings with regular pentagons and hexagons with sides equal to 1.36 A, and external C—NH_2 and C=O bonds of 1.36 and 1.21 A, respectively. The hydrogen-bond distances N—H···O and N—H···N were taken as 2.85 and 3.00 A, respectively, and a maximum deviation of N—H···X from linearity of about 15° was allowed. The dimensions given above are within the limits of error for the published data on cytidine[2] and uracil[3] and the hydrochlorides of adenine,[4] guanine,[5] and histidine,[6] and conform to the hydrogen-bond characteristics of numerous compounds,[7] but it should be pointed out that hydrogen-bond-length variations of as large as ±0.20 A have been observed to occur and that deviations from the regularity of the rings will affect only qualitatively the conclusions reached below.

The base pairs resulting from the above considerations are listed in Table 1. The hydrogen bonds across each pair are indicated by identifying the donor and acceptor sites in each base. Deferring, for the moment, the question of the sugar-phosphate-sugar "backbone" chain between successive bases, each of the twenty-four pairs in Table 1 is a possible pairing scheme in a polynucleotide of that particular composition; but it is doubtful, of course, that all these are of significance in

TABLE 1

GEOMETRICALLY ACCEPTABLE BASE PAIRS IN POLYNUCLEOTIDES

	Base I	Base II	Hydrogen Bonding I-II			Base I	Base II	Hydrogen Bonding I-II	
1.	Adenine	Adenine	d2—a3,	a3—d2	13.	Guanine	Thymine	d1—a1,	a1—d
2.	Adenine	Adenine	d1—a1,	a1—d1	14.	Guanine	Thymine	d1 —a2,	a1—d
3.	Adenine	Adenine	d2—a1,	a3—d1	15.	Guanine	Cytosine	d1—a2,	a1—d2
4.	Adenine	Thymine	d1—a2,	a1—d	16.	Guanine	Cytosine	d1—a2,	d2—a1
5.	Adenine	Thymine	d1—a1,	a1—d	17.	Guanine	Cytosine	d1—a1,	d2—a2
6.	Adenine	Cytosine	d1—a2,	a1—d2	18.	Thymine	Thymine	d —a1,	a1—d
7.	Adenine	Guanine	d1—a1,	a1—d1	19.	Thymine	Thymine	d —a2,	a1—d
8.	Adenine	Guanine	d1—a2,	a1—d3	20.	Thymine	Thymine	d —a2,	a2—d
9.	Guanine	Guanine	d1—a1,	a1—d1	21.	Thymine	Cytosine	d —a2,	a2—d2
10.	Guanine	Guanine	d1—a1,	d2—a3	22.	Thymine	Cytosine	d —a2,	a1—d2
11.	Guanine	Guanine	d1 —a3,	d2—a1	23.	Cytosine	Cytosine	d2—a2,	a2—d2
12.	Guanine	Guanine	d3—a2,	a2—d3	24.	Cytosine	Cytosine	d2—a1,	a1—d2

nature. Pairing 1 occurs in crystals of adenine hydrochloride,[4] pairing 12 is observed in guanine hydrochloride,[5] pairing 18 is found in uracil,[3] and pairings 13, 14, 18, 19, and 20 are like that found in diketopiperazine.[8] Figure 1 shows pairing 1 and represents a possible basis for polyadenylic acid, the two chains being equivalent provided that they run in the same direction. Of interest are those pairs for which the base-nitrogen to sugar-carbon bonds are related, or nearly so, by a twofold axis,

since this then allows the one base to replace the other along one chain, provided that the complementary replacement occurs in the other chain; of more particular interest are pairs of pairs for which the N_{base}—C_{sugar} bonds lie in the same positions relative to each other, allowing additional flexibility in the composition. The Watson-Crick structure combines pairings 5 and 15,[9] which predicts the observed analytical data for DNA from several sources and allows the order of bases in either chain to be completely irregular, provided that the two chains have the biologically pleasing complementary relation to each other.

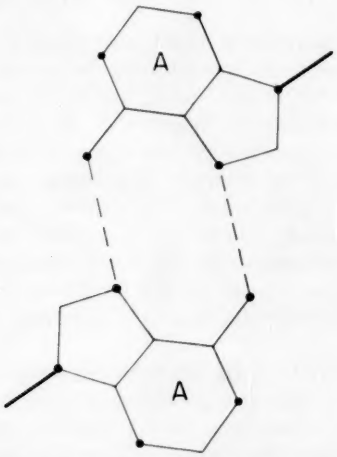


FIG. 1.—Base pairing 1 of Table 1, adenine with adenine. The nitrogen atoms are represented as filled circles and the oxygen atoms as open circles. The hydrogen bonds are the dashed lines, and the bond from the base nitrogen to the sugar is shown as the heavy line.

There is a second pair of pairs, 4 and 17, which has this feature in common with the above. In order to achieve complete equivalence of the N_{base}—C_{sugar} bonds here, however, some distortion from the assumed regular dimensions is necessary. This base-pairing scheme is shown in Figure 2, where it is seen that N_{base}—C_{sugar} bonds are related by a twofold axis parallel to the helical axis, as contrasted to the perpendicular twofold axis in the Watson-Crick formulation.

A third pairing, 6 and 13, shown in Figure 3, is interesting in that it also allows a completely irregular order of all four bases on one nucleotide chain, with the second chain complementary, but in the opposite sense, namely, adenine with cytosine and guanine with thymine. This pairing, like the second, requires some distortion from the regular structures assumed in order to create equivalence of the N_{base}—C_{sugar} bonds.

As shown in Figure 4, a fourth pairing, 2 and 9, is the basis of a two-chain structure having only purine bases, while Figure 5 shows the pairing 18 and 23, which allows only pyrimidines.

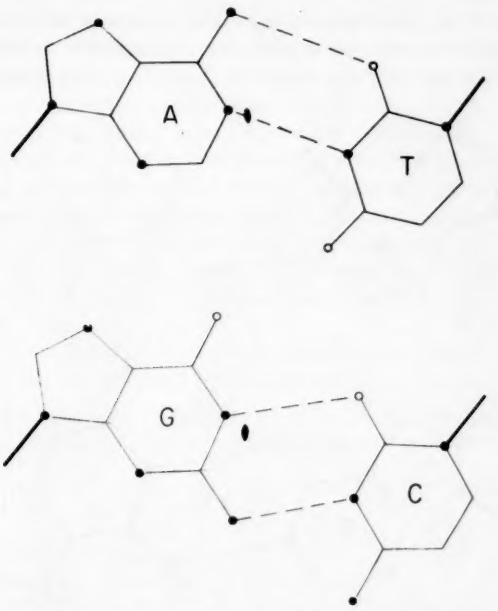


FIG. 2.—Base pairings 4 and 17, adenine with thymine and guanine with cytosine.

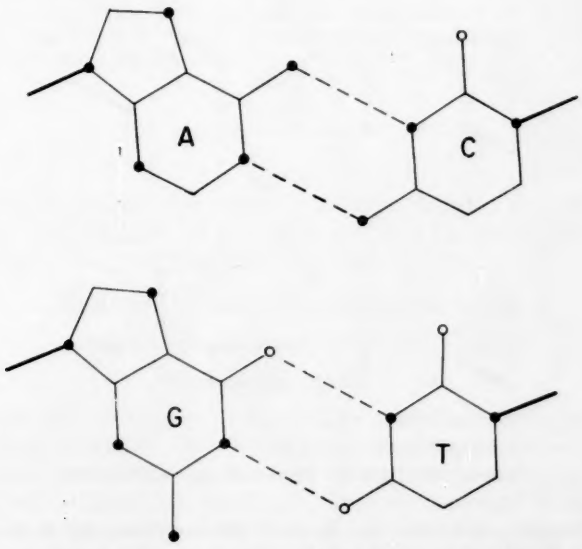


FIG. 3.—Base pairings 6 and 13, adenine with cytosine and guanine with thymine.

Investigation of the above-described pairs of pairs with molecular models shows that it is possible to construct satisfactory right-handed helical polynucleotide structures in which the planes in which the bases lie are 3.4 A apart. For each of

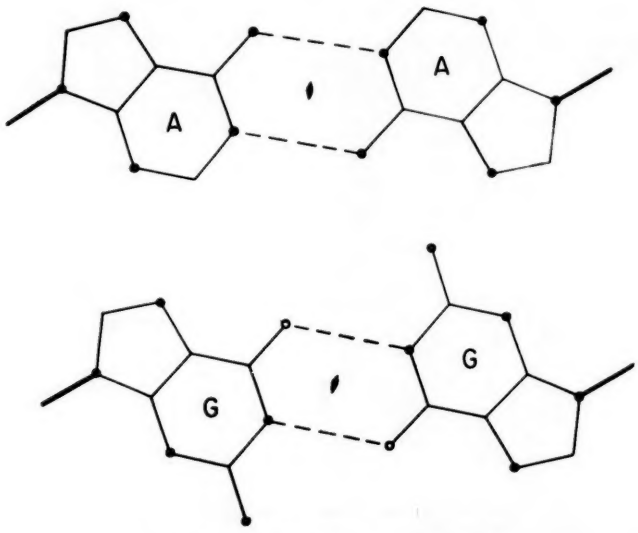


FIG. 4.—Base pairings 2 and 9, purines only.

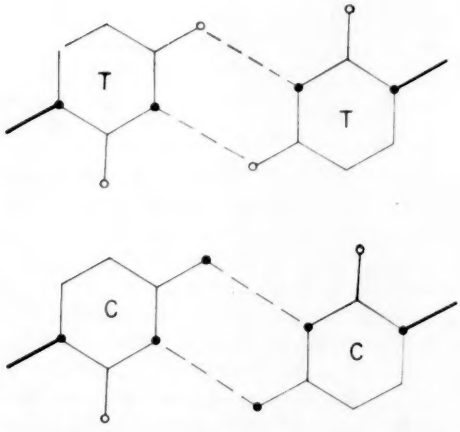


FIG. 5.—Base pairings 18 and 23, pyrimidines only.

them there are two structures: one in which the two chains run in the same direction and one in which they run in opposite directions. For those structures having the twofold axis coincident with the axis of the helix, the repeat along the helical

axis will be halved for the structures with the chain running in the same directions. The angular part of the screw operation of the helix was found to depend largely upon radial co-ordinates of the N_{base}—C_{sugar} bond: for example, for the pairing 4 and 17, in which N_{base} has a radial co-ordinate of 4.5 A, an angular co-ordinate of about 45° gives a satisfactory structure, as contrasted with the Watson-Crick pairing (5 and 15), which has 6.7 A for the N_{base} radius and an angular co-ordinate of 36°, the larger radius requiring the shorter angular separation.

In the absence of more precise information on the bond lengths, bond angles, and particularly the favored orientations to be expected about the numerous single bonds in the polynucleotide chain, it does not seem appropriate to determine exact atomic co-ordinates, especially since there is latitude of ±10° or more in the angular separation of the bases.

We conclude from this study that polynucleotides may assume two-chain structures other than the one proposed by Watson and Crick.

This investigation was aided by a grant from the National Science Foundation.

* Presented at a symposium on "Molecular Structure and Biological Specificity," October 28, 1955, Washington, D.C.

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[8] R. B. Corey, *J. Am. Chem. Soc.*, **60**, 1598, 1938.

[9] It has been pointed out by Professor Pauling that it is possible with only small distortion for guanine and cytosine to pair by formation of three hydrogen bonds (d1—a2, a1—d2, d2—a1), combining pairings 15 and 16. The formation of this additional hydrogen bond may confer extra stability on the Watson-Crick Structure.

PHASE RELATIONS IN SYSTEMS COMPRISING BROMINE AND QUATERNARY AND TERNARY ALKYL AMMONIUM BROMIDES OR CHLORIDES

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Communicated November 28, 1955

The quaternary and ternary alkyl ammonium chlorides and bromides are highly soluble in bromine at 25°. Bowen and Barus[1] have shown that salts of this type complex with bromine molecules to form compounds melting below 60°. However, they did not investigate the phase relations in these systems in a systematic manner. In carrying out measurements of the conductance, viscosity, and vapor pressure of these systems, we found it desirable to determine the phase relations in some detail, in order to establish the nature of the compounds formed.

To establish the phase relation, solubilities were determined by noting the temperature at which the last traces of solid disappeared. A weighed quantity of salt was introduced into a tube which was provided with a stopcock and could be connected to a pump and to a source of bromine. Bromine was distilled into the tube, and the contents were heated until they were homogeneous. The amount of bromine was determined by weighing the tube. The solution was cooled off until crystals appeared. Thereafter, it was slowly heated in a bath, the contents of the tube being mixed by shaking. From time to time the tube was removed briefly from the bath and the solution was examined under a strong light to determine to what extent solid particles remained. Concentrated solutions are transparent, and particles are readily seen if present. The temperature at which the solid particles just disappeared was noted. Thereafter, more bromine was distilled into the tube, and the solubility was again determined.

Phase relations were determined for tetra-*n*-butylammonium bromide, trimethylammonium bromide, trimethylammonium chloride, and tri-*iso*-amylammonium chloride. With the last-named compound the existence of only the complex with one molecule of bromine, $(C_5H_{11})_3NHCl \cdot Br_2$, was established, because the viscosity of the solutions was so high that other compounds could not well be found by the method employed.

Bromine was purified by the method described by Moessen and Kraus.[2] Salts were prepared and purified according to conventional methods. The ternary salts decompose at higher temperatures; Bu_4NBr melted at 117.3–117.7°.

In Figure 1 are shown phase diagrams of three salts, mole fractions of the solutions being plotted against the temperature at which solid particles just disappeared. The plot for Me_3NHCl is displaced with respect to that for the other salts. In Table 1 are given the composition and the melting points for the solid phases that have been identified.

TABLE 1

COMPOUNDS FORMED WITH BROMINE BY DIFFERENT SALTS

Mole Per Cent Br_2	Formula	M.P., ° C.	Mole Per Cent Br_2	Formula	M.P., ° C.
50	$Bu_4NBr \cdot Br_2$	76 (app.)	50	$Me_3NHBr \cdot Br_2$	34
75	$Bu_4NBr \cdot 3Br_2$	37	66	$Me_3NHBr \cdot 2Br_2$	6
50	$Me_3NHCl \cdot Br_2$	37	75	$Me_3NHBr \cdot 3Br_2$	1.5
66	$Me_3NHCl \cdot 2Br_2$	11.5	50	$Am_3NHCl \cdot Br_2$	33
80	$Me_3NHCl \cdot 4Br_2$	11.5	..	.....	..

The complexes formed by the bromide and chloride ions depend on the alkyl groups of the positive ions. Thus Bu_4NBr forms complexes with one and three molecules of bromine, while Me_3NHBr forms complexes with one, two, and three molecules of bromine. On the other hand, Me_3NHCl forms compounds with one, two, and four, but not with three, molecules of bromine.

All the compounds exhibit maximum melting points, and successive phases form eutectics, with the possible exception of $Me_3NHBr \cdot Br_2$. The uncomplexed salt in this case is practically insoluble in the melt. Bu_4NBr is quite soluble in the resulting melt, forming a eutectic with $Bu_4NBr \cdot Br_2$ at 40 mole per cent bromine. Me_3NHCl forms a eutectic with $Me_3NHCl \cdot Br_2$ at about 45 mole per cent bromine. The low solubility of $Me_3NHBr \cdot Br_2$ in the melt is somewhat surprising.

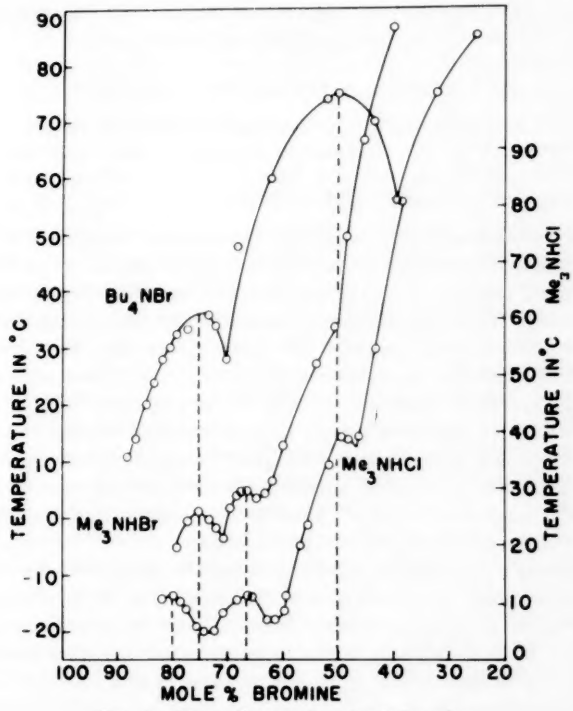

FIG. 1.—Phase diagram for salts in bromine.

No evidence was found indicating exchange between chloride ions and bromine.

* The investigation here reported was supported by the Office of Naval Research under Contract N7onr-35809. This paper is based on a portion of a thesis presented by Philip L. Mercier in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Brown University, June, 1955.

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[2] G. W. Moessen and C. A. Kraus, these PROCEEDINGS, **38**, 1023, 1952.

*CHEMICAL ALTERATION OF CROSSING-OVER FREQUENCY IN CHLAMYDOMONAS**

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Communicated December 2, 1955

Mazia and Ambrose and Gopal-Ayengar have recently suggested that chromosomes are composed of particulate "macromolecular complexes of nucleic acid and proteins. . .linked together by bridges of divalent ions (Ca, Mg, or both)"[1] or by hydrogen bonds.[2] This hypothesis was based on various lines of evidence: that chromosomes disperse when treated with chelating agents;[1] the observations of Steffensen[3] that deficiencies in either calcium or magnesium increase the frequency of chromosome breaks in *Tradescantia;* and Williamson and Gulick's[4] finding that mammalian cell nuclei contain unusually large amounts of calcium and magnesium. In this connection it is of interest that Jungner[5] has found relatively high concentrations of magnesium in purified nucleic acids from several sources and has observed that the magnesium content varied within fairly narrow limits, apparently characteristic of the various nucleic acids examined.

Genetic crossing over[6] would be expected to be intimately related to chromosome structure. Accordingly, an investigation was undertaken of the effects of calcium and magnesium and of treatment with a chelating agent on crossing over in *Chlamydomonas.* This haploid uninucleate green alga is in some ways perhaps better suited experimentally[7, 8] for such studies than is *Drosophila,* in which Levine[9] has recently demonstrated modification of crossing-over frequency by changes in calcium level.

Methods.—Six growth-factor-requiring strains of *Chlamydomonas reinhardi* Stock 137C, representing three pairs of linked genes, were used for the cross-over frequency studies to be reported. The *arg*-1 strain requires arginine, citrulline, or ornithine,[7] and the *arg*-2 strain utilizes arginine only; the two genes responsible for the deficiencies are about 6 map units apart. The *pab*-1 strain will grow only if supplied *p*-aminobenzoic acid, and its requirement is controlled by a gene which is roughly 23 map units from the gene responsible for the nicotinamide requirement of the *nic*-5 strain. The intact thiamine molecule is necessary for growth of the *thi*-1 mutant, and acetate is required for growth of mutant strain *ac*-157b. The *thi*-1 locus is 14 map units from the *ac*-157b locus. For further details on these strains and on culture methods and media see Eversole.[8]

As a preliminary screening technique for exploring conditions and chemical treatments which might influence crossing-over frequency, *in situ* germination of zygotes as described by Eversole[8] was employed, using crosses of mutant strains *arg*-1 by *arg*-2. When treatment altering crossing-over frequency were found, more precise genetic analyses were performed, using the manual manipulation procedure of Ebersold[7] for the isolation of zygotes and in some cases isolation of their four meiotic products.

Three different chemical treatments of cells for one or more hours just prior to mating were investigated for their effects on crossing over. These were (1) ethylenediamine tetraacetic acid (EDTA) extractions, (2) $MnCl_2$ treatment,[10] and (3)

an incubation in high concentrations of calcium and magnesium subsequent to either EDTA or $MnCl_2$ treatment. Yeast extract–acetate agar grown cells were extracted with 10^{-4} *M* EDTA in Petri plates at 20° C. with a 100-watt light bulb at one side. They were permitted to swim under these phototactic conditions for 24–48 hours, except that in certain experiments with *arg*-1 and *arg*-2 the extraction was terminated after 6 hours. At the end of the extraction period the cells were immediately mated on the surface of minimal agar plates. The procedure of Demerec and Hanson[10] for $MnCl_2$ treatment of *Escherichia coli* was followed for cross-over experiments with *Chlamydomonas*. Cells grown on yeast extract–acetate agar were suspended in water for 1–2 hours, in which time they formed flagella and accumulated phototactically. They were centrifuged and washed with 0.019 *M* sodium chloride, the highest concentration which *Chlamydomonas* will tolerate, and incubated for 1 hour in 0.04 per cent $MnCl_2$. Then the cells were mated immediately in the usual manner on minimal agar. The number of reverse mutations produced by this treatment and viability were determined by plate counts on minimal and on supplemented agar media. Treatment was varied in certain experiments by omitting the sodium chloride wash and suspending the cells directly in $MnCl_2$. Under these conditions the cells did not become motile or accumulate phototactically within 1 hour; they were therefore left in $MnCl_2$ for $2^1/_2$–4 hours.

Certain treatments were followed by incubation of the cells in high concentrations of calcium and magnesium. Following the EDTA extractions, 5 ml. of a solution containing 200 p.p.m. Ca ($CaCl_2$) and 200 p.p.m. Mg ($MgCl_2$) were added to 5 ml. of EDTA containing the extracted cells, and the mixture was allowed to stand for several hours before mating. Following the $MnCl_2$ treatment, the cells were centrifuged and resuspended in the same Ca-Mg solution for several hours and then were mated.

Intracellular concentrations of calcium and magnesium were determined on cells with and without extraction with EDTA. The cells were grown for 7–9 days in the light at 25° C. on the surface of yeast extract–acetate agar medium in 500-ml. Erlenmeyer flasks. The cells were scraped off the surface and placed either in distilled water or in 10^{-4} *M* EDTA in Petri plates and allowed to stand at 20° C., with light coming from one side. At the appropriate time a small sample of cells was taken for mating to determine cross-over frequency. The remaining cells were washed twice and dried overnight at 80° C., and the dry weight was determined. Sufficient cells were used to give about 200 mg. of dry cells per crucible. The dry cells were ashed in an oven at 1500°–1550° F. for 6–7 hours. The prolonged ashing time was used as a means of eliminating amounts of sodium, potassium, and phosphate which would interfere with subsequent calcium analyses. After the crucible had cooled in a desiccator and the weight had been determined, the ash was dissolved in concentrated nitric acid and the solution evaporated to dryness. The residue was dissolved in distilled water and diluted to 10 ml., and aliquots were analyzed for calcium and magnesium. The direct EDTA titration method of Gehrke, Affsprung, and Lee[11] for magnesium determination was adapted for use on ashed cells. Oxalate precipitation of calcium and zinc was used to eliminate the small amounts of these interfering substances, and the concentrations of EDTA used for titrations were more dilute (*ca.* $10^{-5}M$). Calcium was determined by direct-flame photometry, according to Kingsley and Schaffert.[12]

Results.—Suitable conditions for EDTA extraction and $MnCl_2$ treatment were established by following recombination by *in situ* germination. Zygotes produced at the same time, but kept in the dark, were then manipulated to fresh minimal agar plates, and in some cases the four meiotic products were separated after zygote germination. Results of experiments with whole isolated zygotes from crosses of strains *arg*-1 by *arg*-2 are shown in Table 1. The *F*-value is a convenient approximate measure of recombination:

$$F = \frac{\text{No. recombination zygotes} \times 100}{\text{Total no. zygotes}}$$

Recombination zygotes (tetratype and nonparental ditype)[13] contain wild-type recombinants and hence grow as wild-type colonies on minimal medium. The values of *F* for *arg*-1 by *arg*-2 zygotes under normal conditions have varied between 6 and 13 in seven experiments involving over one thousand zygotes. When extractions with EDTA were of 24 or more hours' duration, increased *F*-values be-

TABLE 1

RECOMBINATION IN WHOLE ZYGOTES* FROM CROSSES OF *arg*-1 BY *arg*-2

TREATMENT	WILD TYPES	TOTAL	*F*	Mg†		Ca†	
				arg-1	*arg*-2	*arg*-1	*arg*-2
None	...	...	6–13	402	498	645	602
				386	398	505	629
EDTA (39 hr.)	110	145	76.0	164	153	404	523
EDTA (47 hr.)	69	109	63.3	102	139	502	486
EDTA (25 hr.)	49	83	59.0	224	185	490	523
EDTA (6 hr.)	27	90	30.0	...	...	...	...
Then Ca-Mg	4	62	6.4	...	...	...	...
EDTA (6 hr.)	27	88	30.7	...	...	...	...
Then Ca-Mg	5	69	7.3	...	...	...	...
(NaCl) $MnCl_2$	139	179	77.7	...	...	...	...
(NaCl) $MnCl_2$	55	91	60.5	...	...	...	...
(NaCl) $MnCl_2$	48	64	75.0	...	...	...	...
$MnCl_2$	32	121	26.4	...	...	...	...
Then Ca-Mg	9	71	12.6	...	...	...	...
$MnCl_2$	36	99	36.4	...	...	...	...
Then Ca-Mg	8	76	10.5	...	...	...	...

* Zygotes manipulated to fresh agar plates.

† Results expressed in micrograms of Ca or Mg per gram dry weight of cells.

tween 59 and 76 were obtained in three experiments involving 337 zygotes. Following prolonged EDTA extractions and treatment with Ca and Mg, the cells were no longer sexually functional. Zygotes could be produced, however, with EDTA extractions of only 6 hours, followed by Ca-Mg treatment. In these cases *F*-values for the EDTA extraction alone (30.0 and 30.7) were higher than in controls but lower than in experiments involving longer extraction times. Following the Ca-Mg treatment, the *F*-values were 6.4 and 7.3. Since the crossing-over frequency could be restored to the control level by treatment with calcium and magnesium, the EDTA effect appears to be correlated with deficiencies of these metallic ions.

$MnCl_2$ treatment is similar in its effect to that of EDTA. The values of *F* varied from 60.5 to 77.7 in three experiments in which more than 95 per cent of the cells survived the treatment and no reverse mutations were recovered in 10^5 cells. Therefore, all the wild types recovered from crosses are considered to be genetic recombinants and not reverse mutations. After NaCl-washed, $MnCl_2$-treated

cells were incubated in a calcium and magnesium solution, zygote formation failed in the case of *arg*-1 by *arg*-2; however, a reversal of the $MnCl_2$ effect was possible when the NaCl wash was omitted. In this case *F* was also lower (26.4 and 36.4), but Ca-Mg treatment returned the values essentially to the control range (12.6 and 10.5).

Table 2 shows the results of tetrad analysis of zygotes from some of the *arg*-1 by *arg-2* experiments. These findings confirm the results given in Table 1.

TABLE 2

TETRAD ANALYSIS* OF ZYGOTES FROM CROSSES OF *arg*-1 BY *arg*-2

Treatment	PD	T	NPD	*F*	Map Distance	Expected NPD†
None	198	23	3	11.6	6.5	0.31
EDTA (39 hr.)	21	18	8	55.2	36.2	1.08
EDTA (25 hr.)	14	23	9	69.5	44.6	1.91
EDTA (6 hr.)	30	15	2	36.2	20.2	0.67
Then Ca-Mg	41	4	1	10.9	6.5	0.05
(NaCl) $MnCl_2$	15	23	10	68.8	44.8	1.82
(NaCl) $MnCl_2$	34	38	21	63.5	43.0	2.42
(NaCl) $MnCl_2$	10	8	3	58.0	33.4	0.48
$MnCl_2$	29	11	2	30.9	17.9	0.42
Then Ca-Mg	47	4	0	7.9	3.9	0.04

* PD, parental ditype; T, tetratype; NPD, nonparental ditype.

† Calculated as $NPD = T^2/8\,(1 + 2T/3)$; see H. P. Papazian, *Genetics*, **37**, 175, 1952.

Intracellular concentrations of calcium and magnesium were determined in the three EDTA experiments in which the extractions were longer than 24 hours and, for comparison, in two experiments with untreated controls. These results are included in Table 1 and show that magnesium, but not calcium, was significantly lower. On this basis the EDTA effect is associated with a magnesium deficiency. Calcium and magnesium were both significantly lower following prolonged EDTA extraction in cases in which zygote formation failed. Lewin[14] has reported that calcium is required for zygote formation in *C. moewusii*.

Experiments with two additional pairs of gene loci, apparently in different linkage groups, are summarized in Table 3. The cross-over frequency between *pab*-1

TABLE 3

RECOMBINATION IN WHOLE ZYGOTES* FROM CROSSES OF *pab*-1 BY *nic*-5 AND *thi*-1 BY *ac*-157b

	pab-1 BY *nic*-5			*thi*-1 BY *ac*-157b		
TREATMENT	Wild Types	Total	*F*	Wild Types	Total	*F*
None			38–44			25–34
EDTA (24 hr.)	54	88	61.2	29	85	34.1
Then Ca-Mg	38	89	42.7	28	90	31.1
EDTA (26 hr.)	56	88	63.6	26	93	27.9
Then Ca-Mg	42	100	42.0	30	95	31.6
(NaCl) $MnCl_2$	62	92	67.4	23	82	28.0
Then Ca-Mg	33	90	36.7	27	90	30.0
(NaCl) $MnCl_2$	53	82	64.8	26	92	28.3
Then Ca-Mg	28	92	30.4	29	97	29.9

* Zygotes manipulated to fresh agar plates.

and *nic*-5 (F = 38–44) is almost four times greater than that between *arg*-1 and *arg*-2; the effect of either EDTA or $MnCl_2$ treatment would therefore be expected to be less. However, both chemical treatments significantly increased the cross-over frequency. The frequency of recombination between *thi*-1 and *ac*-157b (F =

25–34), although initially less than between *pab*-1 and *nic*-5, was not significantly altered by either EDTA or $MnCl_2$.

The data in Table 2 on crosses between *arg*-1 and *arg*-2 suggest an excess of four-strand double crossovers, as calculated from the observed frequency of tetratypes (T) by equation (5) of Papazian.[15] The appearance of nonparental ditypes appears to be independent of EDTA or $MnCl_2$ treatments. Similar results have been reported for other crosses of *C. reinhardi* involving the *arg*-1 and *arg*-2 linkage group (Ebersold[7]) and for crosses in which the sex locus of *Neurospora crassa* was used as a marker (Barratt *et al.*[13]). Such excesses above the expected ratio of NPD:T could arise from negative chiasma interference, from positive chromatid interference, or from single exchanges at the two-strand stage. Moewus[16] has reported crossing over at the two-strand stage in 100 per cent of the cases when *C. eugametos* zygotes are germinated at low temperatures. However, in the experiments reported here, two-strand-stage crossing over in less than 1 per cent of the cases could account for the observed excesses of nonparental ditypes.

The results reported here show clearly that the ionic environment significantly affects crossing-over frequency in *Chlamydomonas*. The effect of EDTA extraction, shown to result in decreased levels of Ca and Mg, increases crossing-over frequency, in a manner qualitatively similar to that reported by Levine[9] for *Drosophila*, although the quantitative effect in *Chlamydominas* is considerably greater. There would appear to be an additional difference in regard to crossing over, in that the effects of variations of Ca level seem greater in *Drosophila*[9] and of Mg level in *Chlamydomonas*. The difference in behavior of the loci examined in *Chlamydomonas* suggests that different regions of chromosomes of this microorganism may differ in susceptibility to crossing over in relation to the cationic environment. Further work will be required to determine the extent and significance of this variability.

The ionic environment could affect crossing over indirectly, via physiological routes. However, the results reported here and those reported by Levine[9] are consistent with a role of bivalent cations in chromosome structure as proposed by Mazia.[1]

A satisfactory explanation of the $MnCl_2$ effect is more difficult to formulate than that of EDTA. However, a mechanism of action related to that of EDTA is suggested by the reversal of the effects of both chemicals by calcium and magnesium and by the similar responses of different chromosome regions to both chemicals. It is possible that the $MnCl_2$ effect is due to the replacement of some of the Mg in the chromosome by Mn. The chelates of magnesium and manganese are quite similar chemically. The stability constants of the chelates of the two metals are roughly equivalent, but manganese has a slightly greater affinity than magnesium for most organic chelating agents[17] and perhaps for chromosomal constituents.[18]

Summary.—Variations in crossing-over frequency have been shown to follow treatment of cells of biochemical mutant strains of *C. reinhardi* with a chelating agent or with $MnCl_2$. The frequency of crossing over was increased by these treatments in two of three chromosome intervals tested. The effects of both agents were reversed by subsequent incubation of treated cells in high concentrations of calcium and magnesium ions. It is suggested that the action of $MnCl_2$ may involve the replacement of Mg by Mn.

The sensitivity of crossing over in *C. reinhardi* to ionic environment is consistent with the theory that chromosomes are composed of macromolecular unit particles linked together by divalent ions.

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A FURTHER ANALYSIS OF THE FORKED LOCUS IN DROSOPHILA MELANOGASTER

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One question posed by the discovery of pseudoallelism is whether crossing over may be utilized to define the limits of the gene. Alternatively, this question may be stated as follows: Are pseudoallelic mutants divisible by crossing over into a discontinuous array just as are linked, nonallelic mutants, or do pseudoalleles represent a continuous array of chromosomal sites within which crossing over occurs? The data from *Drosophila* are consistent with the first-stated view. However, the putative cases of pseudoallelism recently described in bacteriophage by Benzer[1] and in *Salmonella typhimurium* by Demerec *et al.*[2] suggest that pseudo-

alleles may represent a continuous array of mutational sites separable by recombination. Previously, an analysis of four independent forked (*f*) bristle mutants in *D. melanogaster* showed that each may be assigned to one of two loci as defined by crossing over.[3] The subsequent data represent a continuation in the analysis of the forked locus designed to determine whether more than two loci occur.

Crossing-over Tests.—Six independent *f* mutants were analyzed for recombination. All, when tested to the suppressor of *f* (*su-f*), proved to be unsuppressed. Phenotypically, five are similar to f^1, while one, f^{36a}, produces an extreme forked phenotype. Two mutants, f^h and f^{36a}, arose spontaneously, while the remainder, designated f^{x1}, f^{x2}, f^{x3}, and f^{x4}, occurred following X-irradiation.

Crossing-over experiments were carried out as described previously. In order to maximize crossing over, all *f* mutants were inserted into the scute-8 inversion ($In(1)sc^8$), and ♀ ♀ were made heterozygous for an independent *f* mutant, an *f* tester mutant, as well as the autosomal inversions *Cy* and Ubx^{130}. Only ♂ progeny were scored. Since crossing over occurs between mutants f^1 and f^{3n}, they were used as testers for the *f* loci. The X chromosome of each *f* mutant to be tested carried, in addition, the markers *un* (uneven eye) 1 unit to the right and Bx^2 (Beadex-2 wing) 2.8 units to the left of the *f*. The f^1 tester carried the mutant *B* (Bar eye) 0.3 units to the left of *f*. It should be noted that in $In(1)sc^8$, the f^1 locus occurs to the left of f^{3n}. If a given *f* mutant has its locus to the right of f^1, then recombination types expected on testing to f^1 are $Bx^2\ f^+$ and $Bf^{ex}\ un$ (where f^{ex} is extreme *f*, the phenotype concomitant to two *f* pseudoalleles coexisting on the same X chromosome.) If the *f* locus occurs to the left of f^1, the expected recombination types are $Bx^2\ f^{ex}$ and $B\ f^+\ un$. Similarly, if the *f* mutant has its locus to the left of f^{3n}, the recombination types on testing to f^{3n} are $Bx^2\ f^{ex}$ and $un\ f^+$; however, if the locus occurs to the right of f^{3n}, the expected recombination types are $f^{ex}\ un$ and $Bx^2\ f^+$.

Since all six *f* mutants investigated are unsuppressed, they were first tested for crossing over with f^1, a suppressible mutant. The results are listed in Table 1.

TABLE 1

TESTS OF *f* MUTANTS FOR RECOMBINATION WITH f^1

ALLELE TESTED	N RECOMBINANT ♂♂ $Bx^2\ f^+$	$B\ f^{ex}\ un$	N ♂♂
36a	0	*	77,130
h	0	0	33,823
x1	0	0	45,948
x2	0	2	14,007
x3	2	0	16,399
x4	0	1	11,281

* Not readily detectable, since f^{36a} produces an f^{ex} phenotype.

It may be noted that three of the *f* mutants recombined with f^1, and, on the basis of the distribution of the marker genes among the recombinants, are localized to the right of f^1, presumably allelic to f^{3n}.

Three *f* mutants failed to recombine with f^1. This failure was interpreted to mean that they are truly allelic to f^1. Accordingly, these mutants should recombine with f^{3n}. These tests were made, and the results are listed in Table 2.

Two pertinent facts are evident. First, the *f* mutants which failed to recombine with f^1 all recombined with f^{3n}. Consistent with the distribution of the marker

genes to the recombinants, the *f* mutants are localized to the left of f^{3n}, thereby fulfilling the interpretation, noted above, that they are allelic to f^{1}. Second, no evidence has been uncovered in either test for the existence of *f* loci to the left of f^{1} and to the right of f^{3n}. It would be peculiarly fortuitous for both *f* testers selected to represent the extreme recombinational limits of the loci. Similarly, no evidence is available which points to the occurrence of *f* loci between f^{1} and f^{3n}. While too much significance cannot be placed on the frequency of recombination, it is pertinent, nonetheless, to note that the data are consistent. Thus the recombination frequency manifested by mutants crossing over with f^{1} was 5/41,687, or about 1/8,000, while with mutants crossing over with f^{3n} the frequency was 11/97,419, or about 1/9,000. (The number of recombinants between f^{36a} and f^{3n} was doubled, since only the f^{+} class could be detected.) The totality of the recombination data militate for the interpretation that the six *f* mutants studied here and the four analyzed previously occur in either one of two *f* loci, with five alleles occurring at each locus.

TABLE 2

TESTS OF *f* MUTANTS FOR RECOMBINATION WITH f^{3n}

ALLELE TESTED	*N* RECOMBINANT ♂♂ unf^{+}	$Bx^{2}f^{ez}$	*N* ♂♂
36a	1	*	29,201
h	4	0	24,290
x1	3	2	43,928

* Not readily detectable, since f^{36a} produces an f^{ez} phenotype.

It should be noted that these observations invalidate the hypothesis proposed earlier that separation of the *f* pseudoalleles by crossing over parallels their separation based on their phenotypic response to the suppressor of *f*. Thus suppressed and unsuppressed mutants now prove to be allelic.

Mutation Analysis.—A detailed study of the data reported by Benzer and by Demerec *et al.* leads to the conclusion that essentially each independent mutant analyzed represents a mutation at a separate locus. Thus, in the case of phage, almost every new mutant recombines with all other mutants, and in the case of *Salmonella* each mutant is transduced by nearly all other mutants. This is the basis for the interpretation of a continuous array of pseudoallelic loci. The following represents an attempt to determine from the analysis of induced mutants whether more than two *f* loci are indicated. The rationale of the analysis is predicated on the following observations. First, in all cases observed, the phenotypic consequence of two *f* pseudoalleles coexisting on the same X chromosome is an unsuppressed, f^{ez} phenotype. Second, X-ray-induced mutants producing an f^{1} type or medium-*f* phenotype in general behave as single-locus mutants. The four X-ray-induced mutants reported here support this statement. Third, most X-ray-induced mutants are of the f^{1} type. Fourth, X-ray mutants are of the unsuppressed type. This is borne out by the observation that, of 12 X-ray *f* mutants tested to *su-f*, all were unsuppressed.

From a study of *f* mutants induced in a chromosome already carrying a *f* mutant, contrary results are predictable, depending upon the nature of the *f* locus. The detection of induced mutants is facilitated by irradiating *f su-f* individuals. Induced mutants will occur as either f^{1} or f^{ez} phenotypes among *f su-f* sibs whose phenotype approaches wild type. Following X-irradiation of *f su-f*, if a multi-

plicity of *f* loci occur, f^1-type mutations will occur primarily at loci independent of f^1 and will be manifested as f^{ez} phenotypes. If only two loci occur, two consequences of f^1-type mutants are expected. If mutation occurs at the f^{3n} locus, the resultant individuals will be f^{ez} types. If mutation occurs at the f^1 locus, f^1 will mutate to an unsuppressed type which will be manifested as an f^1 type unaffected by *su-f*. In summary, following irradiation of *f su-f*, if a multiplicity of loci occur, induced mutants will occur as f^{ez} types, and f^1-type mutants will be rare; but if two loci occur, f^1 types will occur as frequently as f^{ez} types.

Two experiments were performed to test the hypotheses. In one experiment, f^+ ♂♂, in the second, *f B su-f* ♂♂, were treated with an X-ray dose of 4,000 r, crossed to attached-X ♀♀, and the ♂ progeny scored for *f* mutants. The results of these experiments are tabulated in Table 3. All mutants listed were tested and

TABLE 3

INDUCTION OF *f* MUTANTS BY X-IRRADIATION

GENOTYPE ♂♂ IRRADIATED	*N* NEW MUTANTS		*N* ♂♂
	f^1 Type	f^{ez} Type	
f^+	4	2	36,651
f^1 *B su f*	3	2	22,721

proved not to be singed bristle mutants which produce a phenotype simulating forked, and in the case of *f B su-f* irradiations were tested and proved not to be reversions of *su-f*. While the data are not voluminous, they point to several conclusions. First, X-irradiation of f^+ indicates that primarily f^1-type mutants are induced. Second, from the irradiation of *f B su-f* as many, if not more, unsuppressed f^1-type mutants were recovered as f^{ez} type, an observation not consistent with the hypothesis of a multiplicity of *f* loci. Since the recombination analysis showed that unsuppressed and suppressed *f* mutants can be alleles, the recovery of unsuppressed medium-*f* mutants following irradiation of f^1 *B su-f* is interpreted to mean that f^1 has mutated to an unsuppressed allele. Therefore, the irradiation analysis failed to uncover any convincing evidence for a multiplicity of *f* loci and is consistent with the crossing-over data.

Discussion.—Taken at face value, the data from *Drosophila* appear to be at variance with the observations made in bacteriophage and *Salmonella*. If it is assumed that the mechanism of recombination in *Drosophila* is identical with that in bacteriophage and with transduction in bacteria, although the available data are not completely compelling on this point, the question may be raised whether an objective comparison is possible at this time. The conclusion that phenotypic alleles are in reality pseudoalleles is based upon the observation that single crossovers occur between these alleles, and the complementary crossover products, wild type and the two mutants coexisting on the same chromosome, are recovered. In the case of the observations in bacteriophage and *Salmonella* the data presented thus far indicate that only "wild-type" progeny have been recovered. Thus the existence of pseudoallelism in these organisms has not been unequivocally demonstrated. Until such a demonstration is made, a comparison of observations must be considered to be conjecture. The recent observations of M. B. Mitchell[4] of so-called "gene conversion" in *Neurospora* and the apparent general occurrence of this phenomenon in microörganisms emphasize the need for detecting both single crossover products before an interpretation of pseudoallelism is warranted. It is

noteworthy to point out that, in the case of the forked pseudoalleles, the Mitchell phenomenon has been ruled out by the recovery simultaneously in attached-X ♀♀ of the complementary products of crossing over between the pseudoalleles.[3]

Contrariwise, it may be argued that in the case of the *f* mutants reported here the failure to uncover more than two loci is related directly to the limited number of mutants tested *inter se*. It should be noted that the recombination data presented demonstrate that no *f* loci occur outside the limits of f^1 and f^{3n} and that no loci occur between the two tester mutants. These conclusions are augmented by observations with the lozenge (*lz*) mutants, where three loci are indicated.[5] Each of the 18 *lz* mutants studied can be assigned, on the basis of recombination, to only one of the three loci, and no additional loci are indicated from the results of extensive *inter se* crossing-over tests.

It might be argued further that the nature of the tester mutants precluded the detection of additional loci. If both f^1 and f^{3n} are associated with minute rearrangements which delimited crossing over so that it could occur only between these mutants, only two loci could be uncovered. There is good reason to believe that neither f^{3n} nor f^1 is associated with any rearrangements. Their cytology is normal. Both mutants have been observed to undergo spontaneous back-mutation to f^+ at characteristic rates, a fact which controverts their association with chromosomal aberration.

It is possible to reconcile the observations in *Drosophila* with those of microörganisms by assuming that two different orders of magnitude are being compared. It may be reasoned that within each *f* locus a number of contiguous, mutational sites occur which, in their linkage relations, are equivalent to the loci described for bacteriophage and *Salmonella*. This viewpoint implies that the locus of the microorganisms is dimensionally equivalent to subdivisions of the loci of *Drosophila* and other higher organisms. The detection of subloci in *Drosophila* would be based on the recovery of recombinations at a rate comparable to that in bacteriophage and bacteria. Experimentation based on rates of recombination of this magnitude is impractical in *Drosophila*.

Summary.—(1) A continuation of the analysis of pseudoallelism at the forked locus in *D. melanogaster* is presented. (2) Crossing over results with ten *f* mutants, as well as mutation experiments at the forked locus, failed to uncover more than two forked loci. (3) These results are discussed in relation to observations on pseudoallelism reported for microörganisms.

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*THE EFFECTS OF THERMAL-NEUTRON RADIATION ON MUTATION OF ENDOSPERM LOCI IN MAIZE**

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This paper presents results of studies of the effects of thermal-neutron radiation on endosperm mutations in maize. Mutations were identified as expressions of the recessive genotype following irradiation of pollen from the dominant type. The principal objectives were (1) to study and compare the rates of mutation of several genetic characters as influenced by different doses of thermal-neutron radiation and (2) to collate these with frequencies of similar mutations induced by X-rays and ultraviolet radiation.

Several studies in which the mutagenic effects of thermal neutrons and X-rays are compared have already appeared in the literature. Of these, the most extensive appear to be those of Conger and Giles[1] on *Tradescantia* and of Caldecott *et al.*[2] on barley.

MATERIALS AND METHODS

Stocks.—Two homozygous inbred tester shocks of *Zea mays* L. were used in these experiments. One of these, BNL 130, carried the dominant genetic loci *A*, *B*, *Pl*, *C*, *R^g*, *Pr*, *Su*, *Sh*, and *Wx*. Pollen of this stock was used for the irradiations. The seed parent, BNL 3, carried the dominant loci *A* and *C* but was recessive *b*, *pl*, *r^g*, *pr*, *su*, *sh*, and *wx*.

Radiation Sources.—Thermal-neutron exposures were made in the thermal column of the Brookhaven National Laboratory nuclear reactor. The cadmium ratio for the facility (ratio of thermal to fast neutrons) was greater than 5,000:1; inherent gamma contamination was about 115 r/hour. X-rays were obtained from a G.E. Maxitron "250" X-ray unit, operated at 250 kvp., 30 ma. The radiation was filtered by 1-mm. aluminum, and the dose rate approximated 800 r per minute. Ultraviolet radiation was from G.E. mercury-vapor (germicidal) lamps in a chamber designed for exposure of pollen from above and below at the same time, under controlled conditions.

Radiation Dosages.—Pollen samples were exposed to doses of thermal neutrons of 3.2, 6.4, 12.8, 19.2, and 25.6×10^{12} N_{th}/cm^2 at a flux of about 9×10^8 $N_{th}/cm^2/sec$. X-ray doses were measured with a Victoreen Integron; dosages were approximately 1,000 1,500, and 2,000 r. Ultraviolet exposures applied were 4.8, 9.6, and 19.2×10^5 ergs/cm^2, given at a dose rate of about 3.2×10^4 ergs/cm^2/sec, as measured with a galvanometer and a phototube. The phototube was exposed only to a small strip of willemite which fluoresced on exposure to light. The ultraviolet output of the lamps was determined as the difference between the energy which passed through ordinary window glass to the willemite and the total light energy which passed through pure quartz.

Procedures.—The tester stocks were grown in fields widely separated from each other. The recessive tester stock, BNL 3, was kept detasseled throughout the entire pollinating season to avoid possible sib contaminations. Pollen for the X-ray and

thermal-neutron exposures was sifted through a fine sieve to remove extraneous material and then placed in small plastic vials. The vials of pollen were then exposed to the radiation. In the ultraviolet radiation treatments a single layer of pollen was carefully sifted onto the center 8 inches of the 12-inch-long, 1-inch-wide quartz slide used in delivering the radiation in the chamber. After exposure, pollen was tapped off the slide to a sheet of paper, and from there the pollen was transferred to the plastic vials used in pollinating. The slide was then wiped clean and the process repeated until enough pollen had been treated.

Pollen samples were collected from individual plants which were identified by a number and the date, in order to exclude the influence of possible spontaneous mutant plants or sectors of plants in the pollen source. No evidence of such influence was found, however, and, in the summary, data for all pollen samples receiving the same radiation doses were combined. All radiation treatments and control pollinations were completed during the period August 8–27, 1953.

The term "mutation," as applied to the endosperm changes produced in this study, is necessarily used in its broadest sense. True gene mutations or point mutations cannot here be distinguished from partial or entire chromosomal losses. It is most probable, however, that the latter classes, including deficiencies, represent the principal types of genetic changes induced.

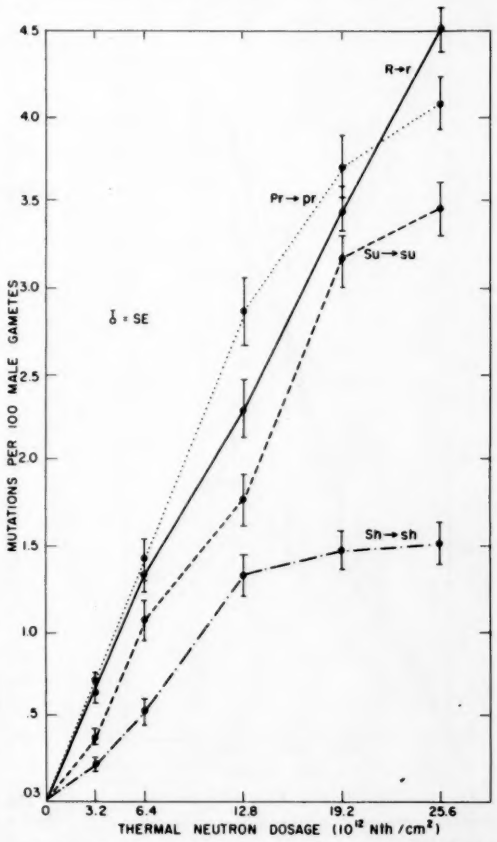

Fig. 1.—The effect of thermal neutron radiation on the mutation of maize endosperm loci (whole endosperm mutations).

RESULTS

The response of endosperm loci *R*, *Pr*, *Su*, and *Sh* to increasing dosage of thermal neutron radiation is shown graphically in Figure 1. For purposes of comparison, the frequencies of similar whole endosperm mutations induced by a limited number

of doses of X-rays and of ultraviolet radiation are shown in Figures 2 and 3, respectively. Since partially mutant endosperms occurred in the radiation treatments, these rates are given along with those for whole endosperm mutants (Table 1). It is evident from the mutation curves that the mutation rates for the endosperm loci are different at corresponding radiation doses.

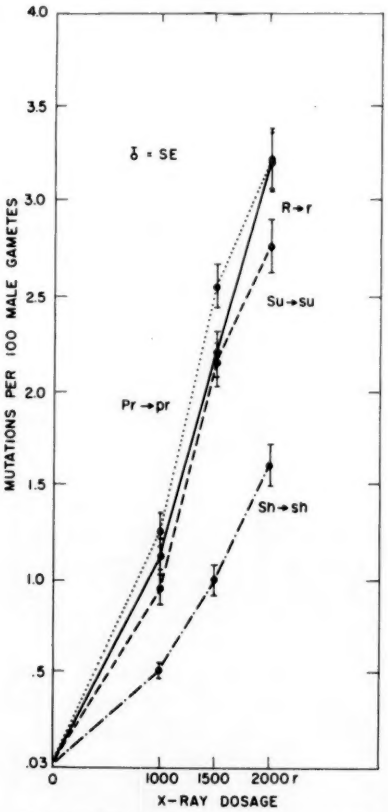

Fig. 2.—The effect of X-rays on mutation of endosperm loci of maize (whole endosperm mutations).

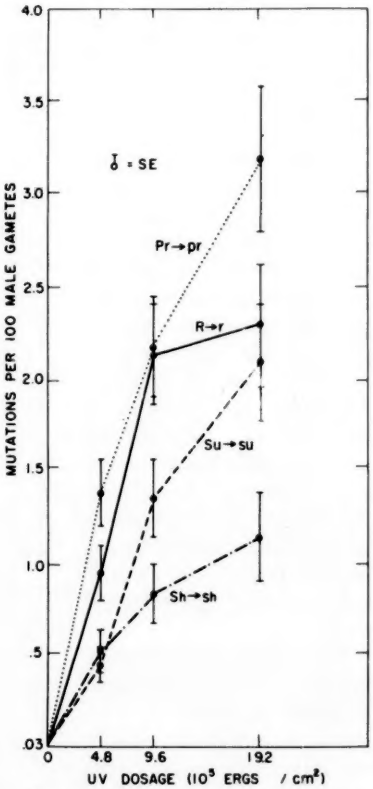

Fig. 3.—The response of maize endosperm loci to ultraviolet radiation (whole endosperm mutations).

The *R* to *r* mutation increases linearly with thermal-neutron dose. No upper limit appears to have been reached for the frequency of these mutants over the dosage range studied. The X-ray data suggest that *R* responds similarly to this radiation, but the ultraviolet data differ in that, at the highest dosage, the mutation of *R* to the phenotype *r* shows a leveling-off. The mutation responses of *Pr* to *pr* and *Su* to *su* are similar for all three radiation types, even though the frequency of *su* endosperms for each dose is slightly lower than that of *pr* endosperms. With the thermal-neutron treatments, a sufficient coverage of the dose-response curve

TABLE 1

EFFECT OF POLLEN IRRADIATION ON PER CENT OF ENDOSPERM MUTATIONS

TREATMENT	ENTIRE KERNEL MUTATIONS						FRACTIONAL KERNEL MUTATIONS					
	r	*pr*	*su*	*shWx*	*shwx*	*shwx* Per Cent of Total *sh* Mutants	*r*	*pr*	*su*	*shWx*	*shwx*	Total Seeds Scored
Control:												
No. obs.	6	6	6	2	1	33	24	53	14	0	1	19,399
Rate*	0.03±0.01	0.03±0.01	0.03±0.01	0.01±0.005	0.005±0.003		0.12±0.02	0.26±0.09	0.07±0.01	0	0.005±0.003	
Thermal neutrons:												
$3.2 \times 10^{12} N_{th}/Cm^2$:												
No. obs.	101	111	61	10	25	74	47	55	39	1	4	15,078
Rate†	0.64±0.06	0.70±0.07	0.38±0.05	0.06±0.02	0.16±0.03		0.19±0.04	0.11±0.03	0.19±0.04	0.07±0.02	0.02	
$6.4 \times 10^{12} N_{th}/cm^2$:												
No. obs.	125	134	100	19	32	64	50	37	57	0	0	9,266
Rate	1.33±0.12	1.42±0.12	1.06±0.11	0.20±0.05	0.34±0.06		0.42±0.07	0.14±0.04	0.55±0.08	0	0	
$12.8 \times 10^{12} N_{th}/cm^2$:												
No. obs.	182	227	141	41	64	61	65	36	67	0	3	7,833
Rate	2.29±0.17	2.87±0.19	1.77±0.15	0.51±0.08	0.81±0.10		0.71±0.09	0.20±0.05	0.79±0.10	0	0.04±0.02	
$19.2 \times 10^{12} N_{th}/cm^2$:												
No. obs.	405	434	372	63	109	64	143	87	128	0	11	11,622
Rate	3.46±0.17	3.70±0.18	3.17±0.16	0.53±0.07	0.93±0.09		1.11±0.10	0.49±0.07	1.03±0.09	0	0.09±0.03	
$25.6 \times 10^{12} N_{th}/cm^2$:												
No. obs.	692	625	530	85	147	64	224	91	194	0	16	15,181
Rate	4.53±0.17	4.09±0.16	3.46±0.15	0.55±0.06	0.96±0.08		1.36±0.09	0.34±0.05	1.21±0.09	0	0.10±0.03	
X-rays:												
1,000 r:												
No. obs.	196	219	168	23	68	76	91	61	66	1	10	17,054
Rate	1.12±0.08	1.25±0.09	0.96±0.08	0.13±0.03	0.39±0.05		0.41±0.05	0.10±0.02	0.32±0.04	0.005±0.004	0.05±0.01	
1,500 r:												
No. obs.	359	417	354	46.0	117	72	186	89	125	1	17	16,223
Rate	2.18±0.12	2.54±0.12	2.15±0.11	0.27±0.04	0.72±0.07		1.03±0.08	0.29±0.04	0.70±0.07	0.006±0.004	0.10±0.08	
2,000 r:												
No. obs.	429	430	368	55	161	75	170	111	151	3	17	13,205
Rate	3.20±0.16	3.23±0.16	2.76±0.14	0.41±0.05	1.21±0.10		1.17±0.09	0.58±0.07	1.07±0.09	0.02±0.01	0.12±0.03	
Ultraviolet:												
4.8×10^8 ergs/cm²:												
No. obs.	39	56	20	5	16	77	81	31	68	7	6	3,996
Rate	0.95±0.15	1.37±0.18	0.45±0.10	0.12±0.05	0.40±0.10		1.91±0.22	0.52±0.11	1.63±0.20	0.18±0.07	0.14±0.06	
9.6×10^8 ergs/cm²:												
No. obs.	61	62	39	9	15	63	75	18	58	1	3	2,830
Rate	2.12±0.27	2.16±0.28	1.35±0.22	0.31±0.10	0.53±0.14		2.53±0.30	0.38±0.11	1.98±0.26	0.04±0.04	0.10±0.06	
19.2×10^8 ergs/cm²:												
No. obs.	46	63	42	6	17	74	76	31	83	1	5	1,980
Rate	2.29±0.34	3.17±0.40	2.09±0.31	0.29±0.12	0.85±0.21		3.72±1.37	1.37±0.26	4.12±1.44	0.05±0.05	0.25±0.11	

* Mutations per 100 male gametes.

† All rates are net, i.e., observed rate *minus* control rate.

was obtained to show that for these two loci there is an apparently linear response of mutations to radiation dosage, but only up to a point. After that, a leveling-off occurs and the curve apparently becomes nonlinear. A similar response is shown to a more marked degree in the thermal-neutron dose curve for mutations of *Sh* to *sh*. Here the plateau of the curve begins at a lower dosage level than for the other loci, a result which may be significantly correlated with the generally lower relative mutation rate of *Sh*. The X-ray curve, which covers a narrower range of dosage, does not show a plateau, so that if it occurs, the leveling-off begins at a dosage level higher than those used in these experiments.

Endosperms showing the recessive phenotype of *Sh* were checked for the expression of *wx* by testing the endosperm tissue with an iodine solution. In this test the endosperm tissue stains blue if starchy, red if waxy. The results are given in the sixth column of Table 1. The data show that as many as 75 per cent of the *sh* endosperms were also *wx*. Shrunken endosperms in the thermal-neutron treatments were less frequently waxy than in the X-ray or ultraviolet treatments.

DISCUSSION

Our mutation tests show that thermal-neutron radiation is an effective mutagenic agent. This is evident from the data of other workers.[1-3] The effects of the different types of radiations studied here appear to be remarkably similar. Such close similarities were not anticipated. However, certain dissimilar effects were observed, and they are noteworthy. It was found that different genetic factors respond quite differently to radiation. In order to discuss this phenomenon with adequate data, the thermal-neutron results will be more fully examined. For example, the mutation of the endosperm phenotype *R* to *r* appeared to increase linearly with thermal-neutron dose. Other endosperm mutations, while showing a similar linear response to lower levels of thermal-neutron radiation, showed smaller increments of mutation at the higher dosages, suggesting a "saturation" effect. This was particularly true for the endosperm factor *shrunken* (*Sh*). For this locus the frequency of mutations increased only slightly beyond the level reached with 12.8×10^{12} N_{th}/cm^2, even with double the radiation dose (Fig. 1). The "saturation" effect on mutation occurs at relatively low dosage levels for one genetic factor, at relatively higher levels for others, and not at all over the dosage range studied for another. This reduction in the rate of increase of one type of endosperm mutation with radiation dose at the same time that the rate of another continues unchanged would appear to indicate some basic difference in the breakability of chromosomal regions.

But could selection account for the unequal response of the different genetic factors to radiation? Apparently not. The mature maize pollen contains the two sperm nuclei, which do not divide further until they enter the egg sac of the female gamete and effect fusions. Experiments with *Lilium* have shown that levels of radiation comparable to those used in the present experiments have little or no influence on the ability of treated pollen to function. This was true in the *Lilium* experiments, in spite of the fact that the sperm nuclei are formed after treatment and during pollen-tube growth.[4] With maize, the endosperm develops rather independently of the embryo. The effect of genetic damage to the sperm is greatly buffered in the endosperm by the two untreated chromosome sets of the polar

nuclei contributed by the female parent, and one would expect that unions in which even fairly large chromatin losses have occurred would prove viable. Weatherwax[5] discussed this earlier. Thus the possible inviability of any one type of mutant is an unlikely explanation of the different mutation responses observed. It may be presumed that most of the changes studied here are due to chromosomal or genic deficiency, since cytological studies indicate that this is the case for most X-ray- and many ultraviolet-induced mutations.[6] The waxy character of the starch in most of the shrunken endosperm mutants suggests that the chromosome deficiency induced is quite large and probably involves the loss of the entire short arm of the chromosome (Table 1, sixth column). It has been indicated that heterochromatic regions of chromosomes are more sensitive to X-ray and ultraviolet breakage than are euchromatic regions.[7] Thus the fact that the centromere region of chromosome 9 is bounded by heterochromatin may account for the greater frequency of breaks produced between the centromere and *Wx* as compared with breaks produced distal from that locus. Similar correlation of greater X-ray breakability with heterochromatic regions was also noted in maize by Longley[8] and in *Drosophila* (see Hannah[9]). The slight difference between the frequency of waxy shrunken mutants in the thermal-neutron and X-ray or ultraviolet treatments may or may not indicate qualitative differences between the biological effects of these radiations. In fact, it is surprising that ultraviolet radiation, which is believed to produce its genetic effects through photon absorption by nucleic acid of the chromosome, most frequently breaks the same regions that are broken most frequently by X-rays.

Previous studies have indicated that chromosome breaks or mutations are linearly related to thermal-neutron dose.[1, 2] The present data agree with this only in part, since the response obtained with certain genetic factors is linear through the range of the lower dosages but becomes nonlinear as the range is extended. Similarities and differences in the response of linked endosperm factors to X-rays, thermal neutrons, gamma rays, and ultraviolet have been observed in a study reported in another paper.[10] Mutation curves obtained in studies with maize that was continuously exposed to gamma radiation also possess similarities to those presented here but suggest that there may be a curvilinear mutation-dose response between certain lower dosage levels.[11] Experiments with developing pollen exposed to gamma radiation show that the same genetic factors that are studied here also vary in their mutability at different stages in gametogenesis.[12]

SUMMARY

The mutation response of endosperm factors *R*, *Pr*, *Su*, and *Sh* to thermal-neutron dosage was studied. The data obtained were compared with those from similar experiments using X-rays and ultraviolet radiation. Individual differences in the mutational responses of the genetic factors were observed, but the similarity in the responses of these factors to three very different radiations studied was especially noteworthy.

* Research carried out at Brookhaven National Laboratory under the auspices of the United States Atomic Energy Commission.

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*THE THERMAL CLASSIFICATION OF LAKES**

BY G. E. HUTCHINSON AND H. LÖFFLER†

Communicated December 16, 1955

Forel[1] introduced, for holomictic lakes of sufficient depth to exhibit thermal stratification, a threefold classification in which the following categories were recognized: *polar* lakes, never at any point over 4° C., inversely stratified under ice in winter and circulating in summer; *temperate* lakes, inversely stratified in winter, directly stratified in summer, and circulating twice a year at about 4° C.; and *tropical* lakes, always at every point above 4° C., circulating in winter and directly stratified in summer.

The terminology has been long considered inappropriate, as many of the finest examples of Forel's tropical type occur in western Scotland and in British Columbia, regions far outside the tropics, while, of the few polar lakes studied, more occur at high altitudes in temperate and tropical latitudes than within the Arctic Circle. It has also become apparent, since the magnificent work of Ruttner[2] in Indonesia, that the typical lake of the humid tropics at low altitudes circulates rarely, in an irregular manner, not at a specific season but when exceptional periods of cool weather permit sufficient heat loss at the surface to cause instability. This presumably happens at intervals of length greater than that of a year.

Recently one of us (H. L.) has had the opportunity to study lakes in equatorial latitudes but at great altitudes, in the Andes. Although at low altitudes in the humid tropics small temperature gradients can maintain stable stratifications, no stable stratification develops at the low temperatures of high altitudes, where the density difference per degree centigrade is very small. The lack of seasonal variation, that permits almost perennial stratification at low altitudes in equatorial latitudes, thus permits perennial circulation at high altitudes in the same latitudes.

We propose for these two types of equatorial lake the terms *oligomictic* and *polymictic*, respectively. For the three categories of Forel's classification the terms *cold monomictic*, *dimictic*, and *warm monomictic* have been used by one of us (G. E. H.) in a forthcoming extensive work on limnology.[3] The theoretical scheme (Fig. 1) may be completed by recognizing for the very rare perennially ice-covered

lakes of the antarctic and, under special conditions, of some high mountains (Löffler unpublished) a category of *amictic* lakes, perennially sealed off by ice from most of the annual seasonal variations in temperature.[4]

It is evident from the considerable body of work on Central Africa that in the dry

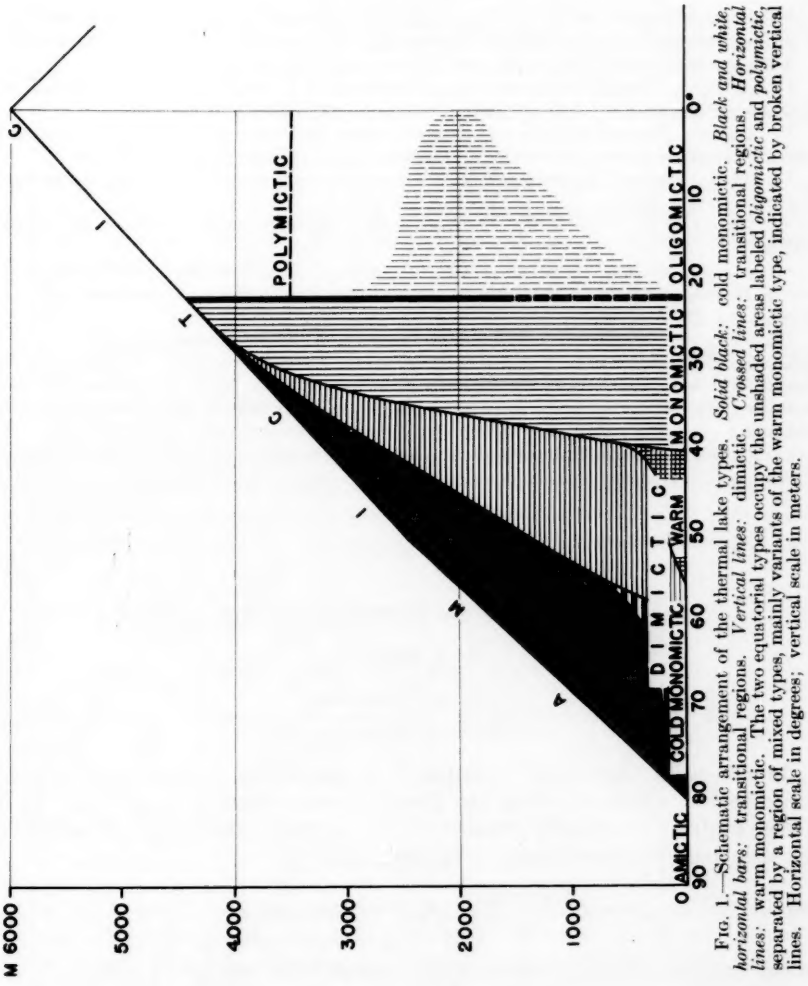

FIG. 1.—Schematic arrangement of the thermal lake types. *Solid black:* cold monomictic. *Black and white, horizontal bars:* transitional regions. *Vertical lines:* dimictic. *Crossed lines:* transitional regions. *Horizontal lines:* warm monomictic. The two equatorial types occupy the unshaded areas labeled *oligomictic* and *polymictic*, separated by a region of mixed types, mainly variants of the warm monomictic type, indicated by broken vertical lines. Horizontal scale in degrees; vertical scale in meters.

equatorial regions of that continent at fair elevations a great variety of conditions occur, linking the oligomictic, with a strong tendency to meromixcis, as in Lake Nyasa,[5] the warm monomictic, as in Lake Mohasi, Ruanda,[6] and the polymictic, as in Lake Tana,[7] Lake Rudolf, Lake Albert, and, to a large extent, Lake Victoria and Lake Naivasha.[8] The existence of special climatic regimes, notably the

monsoonal regime of India and Ceylon, probably produces considerable disturbance of the scheme; Dr. S. Dillon Ripley, for instance, tells us that in Ceylon artificial lakes at an altitude slightly over 2,000 meters may occasionally freeze, though such lakes are certainly not polymictic and presumably belong in the dimictic category.

Further details will be found in our forthcoming independent works.

* This paper is dedicated to Professor Alexander Petrunkevitch on his eightieth birthday. Contribution from the Osborn Zoölogical Laboratory, Yale University, New Haven, Connecticut.

† Theresa Seessel fellow, Yale University, from the University of Vienna.

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GAMMA FACTORS IN FUNCTIONAL EQUATIONS

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In a so-called "functional" equation for a zeta function pertaining to an algebraic field or a modular setup, the Dirichlet series occurring is multiplied by a function $\Delta(s)$ of the complex variable $s = \sigma + i\tau$ which introduces itself each time by some (multiple Euler) integral of the form

$$\frac{\Delta(s)\ \lambda(x)}{\mu(x)^s} = \int_P e^{-(x,\,t)}\ R(t)^s\ d\Omega(t), \tag{1}$$

which each time, by an appropriate computation, turns out to be a product

$$\prod_{m\,=\,1}^{N} \Gamma\ (p_m s + q_m),$$

in which p_m are positive rational numbers and q_m are complex numbers.[1] After replacing $\Delta(s)$ by $\Delta(rs)$ for a suitable positive integer r, the numbers p_m may be assumed to be positive integers themselves, and, applying, now, the classical

formula

$$\Gamma(pw) = C_p e^{D_p w} \prod_{m=1}^{p} \Gamma\left(w + \frac{m-1}{p}\right), \qquad w = s + \frac{q}{p},$$

to each factor, the total product becomes

$$A e^{as} \prod_{m=1}^{n} \Gamma(s - a_m), \tag{2}$$

where

$$A > 0, \qquad a = a \text{ real number}, \qquad a_m = a \text{ complex number}. \tag{3}$$

We are going to give a criterion by which a function $\Delta(s)$ which satisfies relation (1) is indeed of the form (2). The integral (1) which we will envisage will be rather general and will very amply include all the particular cases known. But ours will be a pure existence theorem, and none of the particular computations is in any way superseded by it.

We assume that $x = (x_1, \ldots, x_k)$, $t = (t_1, \ldots, t_k)$ are points in some real Euclidean E_k, $k \geq 1$, and (x, t) is the value $x_1 t_1 + \ldots + x_k t_k$. The point set P is an arbitrary open subset of E_k: (t), and $\Omega(A)$ is a positive set function which is defined on every Borel set A of P whose closure in E_k is a compact subset of P and which is countably additive on any such compactifiable subset. The function $R(t)$ is a (nonhomogeneous) *polynomial* of some (*unprescribed*) degree in $(t_1, \ldots, t_k)$ and is positive on P. The functions $\lambda(x)$, $\mu(x)$ are defined real-valued and infinitely differentiable (actually, the infinite differentiability would follow from the other assumptions put together) over a neighborhood G of E_k: (x) no matter how small and they are positive on G. The complex variable s ranges over some right half-plane

$$\sigma > \sigma_0, \tag{4}$$

and

$$\mu(x) = e^{\log \mu(x) \cdot s}, \qquad R(t)^s = e^{\log R(t) \cdot s},$$

for real values of the logarithms, and $\Delta(s)$ is holomorphic in (4). We also assume that, for

$$x \text{ in } G, \qquad s \text{ in (3)}, \tag{5}$$

we have

$$\int_P e^{-(x,t)} R(t)^\sigma \, |d\Omega(t)| < \infty \tag{6}$$

uniformly for x in G, which implies that integral (1) is holomorphic in (4), and the over-all assumption is that in (4) equality (1) holds.

THEOREM. *Such a function* $\Delta(s)$ *is indeed of the form* (2), (3).

Replacing s by $s + 1$ in equation (1), we obtain

$$\frac{\Delta(s+1)\,\lambda(x)}{\mu(x)^{s+1}} = \int_P e^{-(x,t)} R(t)\, R(t)^s \, d\Omega(t)$$

$$= R\left(-\frac{\partial}{\partial x_1}, \ldots, -\frac{\partial}{\partial x_k}\right) \frac{\Delta(s)\,\lambda(x)}{\mu(x)^s},$$

and it is not hard to see that the right side can be written as an expression of the form

$$\frac{\Delta(s)}{\mu(x)^s} \sum_{m=0}^{n} c_m(x) s^m ,$$

in which $c_m(x)$ are certain functions of x. We thus obtain

$$\Delta(s+1) = \Delta(s) \sum_{m=0}^{n} b_m(x) s^m,$$

and it follows that the $b_m(x)$ are independent of x. After decreasing the integer n, if necessary, we may therefore put

$$\Delta(s+1) = \Delta(s)\, B_0 \prod_{m=1}^{n} (s - a_m), \tag{7}$$

where $\{a_m\}$ are complex numbers and $B_0 \neq$ is real. By considering large positive values of s, it follows that $B_0 > 0$, and thus $B_0 = e^a$, where a is real. It follows, now, from equation (7) that we can put

$$\Delta(s) = e^{as} \prod_{m=1}^{n} \Gamma(s - a_m) \cdot \Delta_0(s),$$

where $\Delta_0(s)$ is, to start with, holomorphic in some half-plane $\sigma > \sigma_1$ and periodic

$$\Delta_0(s+1) = \Delta_0(s).$$

Therefore,

$$\Delta_0(s) = \sum_{r=-\infty}^{\infty} c_r e^{2\pi i r s} \tag{8}$$

everywhere. However, $\Delta(s)$ is bounded in $\sigma_0 + 1 < \sigma < \sigma_0 + 2$, $-\infty < \tau < \infty$, and, by a known property of gamma functions, we have an estimate

$$\prod_{m=1}^{n} \Gamma(s - a_m)^{-1} = O(e^{\varepsilon|t|})$$

in the same strip. Therefore,

$$\Delta_0(s) = O(e^{\varepsilon|t|})$$

throughout; but if a Laurent series $\sum_{-\infty}^{\infty} c_r w^r$ is convergent for $w \neq 0$ and is $O(|w|^{\pm\varepsilon})$ as $w^{\pm 1} \to \infty$, it must be a finite sum. Therefore,

$$\Delta_0(s) = \sum_{r=-g}^{g} c_r\, e^{2\pi i r s}. \tag{9}$$

If in this sum more than one coefficient is $\neq 0$, $\Delta_0(s)$ has zeros which recur periodically. But this implies the existence of a number s_0 for which $\Delta(s_0) = 0$. Relation (1) then implies

$$\int_P e^{-(x,\,t)}\, R(t)^{s_0}\, d\Omega(t) = 0$$

for x in G. By analytic continuation with regard to x and subsequent application of the uniqueness theorem for (multiple) Laplace transforms, this implies

$$R(t)^{s_0}\, d\Omega(t) = 0$$

identically in t, which, in its turn, implies the same relation for all s, and hence $\Delta(s) = 0$, which in our case is contradictory. Thus $\Delta_0(s)$ reduces to a single term. However, the constants a_m are part real and part complex conjugate in pairs, and for real s everything must be real. Hence $\Delta_0(s) = c_0$, which completes the proof of the theorem.

[1] For most recent occurrences compare Max Koecher, "Ueber Dirichlet-Reihen mit Funktional-Gleichung," *J. reine u. angew. Math.*, **192**, 1–23, 1953, and Horst Leptin, "Die Funktionalgleichung der Zetafunktion einer einfachen Algebra," *Abhandl. math. Seminar Univ. Hamburg*, **19**, 198–220, 1955.

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*THE REPRESENTATION OF A C-BIMEASURE ON A GENERAL RECTANGLE**

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The authors are developing a theory of $\boldsymbol{C}$-bimeasures Λ on the product $E' \times E''$ of two locally compact topological spaces. An abstract on "Bimeasures and Their Integral Extensions" will shortly appear in a volume dedicated to M. Picone. A preliminary basis is found in a paper on "Semi-normed Vector Spaces with Duals of Integral Type," to appear this fall in the *Journal d'analyse mathématique*. Papers on "$\boldsymbol{C}$-Bimeasures Λ and Their Superior Integrals" and on "$\boldsymbol{C}$-Bimeasures and Their Integral Extensions" will follow. This note refers to the case where E' and E'' are intervals and establishes the general theorem necessary to make a proper connection with the case where E' and E'' are locally compact topological spaces.

1. *Definitions and Notation.*—Let E' be an interval of the s-axis of one of the forms $[0, a)$, $(0, a]$, $(0, a)$, $[0, a]$. Let E'' be an interval of the t-axis of one of the forms $[0, b)$, $(0, b]$, $(0, b)$, $[0, b]$. Let k, with values $k(s, t)$, map $E' \times E''$ into $\boldsymbol{C}$, the space of complex numbers. The authors have defined the Fréchet variation $P_{\boldsymbol{C}}(k, E' \times E'')$ as follows:

Definition of $P_{\boldsymbol{C}}(k, E' \times E'')$: Let π' be a partition of E' by vertices

$$s_0 < s_1 < \ldots < s_{m'} \tag{1.1}$$

in E', and let π'' be a partition of E'' by vertices

$$t_0 < t_1 < \ldots < t_{m''} \tag{1.2}$$

in E''. For $0 < r \leqq m'$ and $0 < n \leqq m''$, set

$$\Delta_{rn} = k(s_r, t_n) - k(s_{r-1}, t_n) - k(s_r, t_{n-1}) + k(s_{r-1}, t_{n-1}), \tag{1.3}$$

and introduce complex numbers η_r and ζ_n with absolute value at most 1. Set

$$P_{\boldsymbol{C}}(k, E' \times E'') = \sup \left| \sum_{r=1}^{m'} \sum_{n=1}^{m''} \eta_r \zeta_n \Delta_{rn} \right|, \tag{1.4}$$

taking the sup over all possible partitions π' and π'' of E' and E'', respectively,

and all choices of the numbers η_r and ζ_n with $|\eta_r| \leqq 1$, $|\zeta_n| \leqq 1$. The value of $P_{\boldsymbol{C}}(k, E' \times E'')$ may be infinite.

Regular k: Let s_1 and t_1, respectively, be values on the intervals E' and E''. The mapping of E'' into $\boldsymbol{C}$ defined by $k(s, t)\,|\,(s = s_1)$ will be called the s_1-*section* of k and will be denoted by $k(s_1, \cdot)$. The t_1-section of k, $k(\cdot, t_1)$, is similarly defined. If $I'[I'']$ is a subinterval of $E'[E'']$, by the Fréchet variation of k over $I' \times I''$, $P_{\boldsymbol{C}}(k, I' \times I'')$, we shall mean the Fréchet variation of the restriction of k to $I' \times I''$.

We term k *variationally admissible* if (1) the Fréchet variation of k over any closed subrectangle $I' \times I''$ of $E' \times E''$ is finite and (2) for any $s_1 \in E'$ $[t_1 \in E'']$ the Jordan variation of the $s_1[t_1]$ section of k is finite over any closed subinterval of $E''[E']$. The function k is termed *left-left continuous* over $E' \times E''$ if for each point t_0 of E'', $k(\cdot, t_0)$ is left-continuous at each $s \in E' \cap [0, a)$, and if for each point s_0 of E', $k(s_0, \cdot)$ is left-continuous at each point $t \in E'' \cap [0, b)$. A k which is variationally admissible and left-left continuous is termed *regular*. A regular k will be said to have *null lines* $s = s_1$ and $t = t_1$ if $k(s_1, \cdot)$ and $k(\cdot, t_1)$ vanish identically.

In the interest of compactness of notation, we shall depart somewhat from the Bourbaki usage and let $\mathcal{E}'[\mathcal{E}'']$ denote the space of continuous mappings of $E'[E'']$ into $\boldsymbol{C}$ which vanish outside of some closed subinterval of $E'[E'']$. If $I'[I'']$ is a subinterval of $E'[E'']$, we denote by $\dot{\mathcal{g}}'[\dot{\mathcal{g}}'']$ the subspace of $\mathcal{E}'[\mathcal{E}'']$ consisting of functions which vanish outside of $I'[I'']$.

With each bounded $u \in \boldsymbol{C}^{E'}$ $[v \in \boldsymbol{C}^{E''}]$ we associate a norm value

$$U(u) = \max_{s \in E'} |u(s)| \qquad [U(v) = \max_{t \in E''} |v(t)|].$$

A $\boldsymbol{C}$-measure α on E' is then a $\boldsymbol{C}$-linear form on $\mathcal{E}'$ which satisfies the following condition: If I' is any closed subinterval of E', the restriction of α to the subspace $\dot{\mathcal{g}}'$ of $\mathcal{E}'$ is continuous in the topology defined by the above norm. A $\boldsymbol{C}$-measure on E'' is similarly defined.

A $\boldsymbol{C}$-bimeasure Λ on $E' \times E''$ is a $\boldsymbol{C}$-bilinear form on $\mathcal{E}' \times \mathcal{E}''$ with values $\Lambda(u, v)$ such that, for fixed $u \in \mathcal{E}'$ $[v \in \mathcal{E}'']$, the mapping $\Lambda(u, \cdot)$ $[\Lambda(\cdot, v)]$ of $\mathcal{E}''[\mathcal{E}']$ into $\boldsymbol{C}$ is a $\boldsymbol{C}$-measure on $E''[E']$. With a $\boldsymbol{C}$-bimeasure Λ a norm $\Lambda \rightarrow \|\Lambda\|$ may be associated by setting

$$\|\Lambda\| = \sup \frac{|\Lambda(u, v)|}{U(u)\ U(v)}, \tag{1.5}$$

where the sup is taken over all $(u, v) \in \mathcal{E}' \times \mathcal{E}''$ for which $U(u)\ U(v) > 0$. More generally, if Γ is a $\boldsymbol{C}$-bilinear form on $\mathfrak{A} \times \mathfrak{B}$, where $\mathfrak{A}$ and $\mathfrak{B}$ are vector subspaces, respectively, of $\boldsymbol{C}^{E'}$ and $\boldsymbol{C}^{E''}$, of bounded mappings, we shall again denote the corresponding sup (1.5) by $\|\Gamma\|$, taking this sup over all $(u, v) \in \mathfrak{A} \times \mathfrak{B}$ with $U(u)U(v) > 0$. When $\|\Gamma\|$ is finite, we say that Γ is *bounded*.

If Λ is a $\boldsymbol{C}$-bimeasure on $E' \times E''$, and E' and E'' are closed intervals, $\|\Lambda\|$ is finite, but for general intervals E' and E'', $\|\Lambda\|$ need not be finite. However, if I' and I'' are closed subintervals of E' and E'', respectively, and Λ is a $\boldsymbol{C}$-bimeasure on $E' \times E''$, the restriction of Λ to $\dot{\mathcal{g}}' \times \dot{\mathcal{g}}''$ defines a bounded $\boldsymbol{C}$-bilinear form on this space.

2. *Representation of $\boldsymbol{C}$-Bimeasures.*—The objective of this note is the proof of Theorem 2.1 below. In the case in which E' and E'' are both closed intervals, the

proof is essentially contained in Theorem 12.1 of an earlier paper,[1] so that a proof of the theorem involves its extension from the closed-interval case to the general case. While the proof of Theorem 12.1 is given for real bimeasures, the extension of the proof for $\boldsymbol{C}$-bimeasures involves little more than a reinterpretation of the absolute-value sign.

The following lemmas will be of use in the proof of Theorem 2.1.

LEMMA 2.1. *If (1) E' is an interval of the above type, (2) g is a complex-valued function on E', left-continuous except at most at the right end point of E' when E' is closed on the right, (3) for every $u \in \mathcal{E}'$ the Riemann-Stieltjes integral $\int_{E'} u\, dg$ exists and is equal to zero, then g is constant on the interval E'.*

The proof of this lemma, along the lines of the proof of Lemma 4.2 of another paper,[2] is evident and will be omitted.

LEMMA 2.2. *Let E' be an interval of the above type, and let $\dot{\mathcal{E}}'$ be the space of functions continuous on $[0, a]$ which vanish outside of E'. Let λ be a $\boldsymbol{C}$-linear form on $\dot{\mathcal{E}}'$ bounded in the sense that*

$$\sup \frac{|\lambda(u)|}{U(u)} = \|\lambda\| < \infty \qquad [u \in \dot{\mathcal{E}}']. \tag{2.1}$$

There then exists a unique function $g = g_\lambda$ defined on $[0, a]$, left-continuous on $[0, a)$, with $g(0) = 0$, such that

$$\lambda(u) = \int_0^a u(s)\, dg(s) \qquad [u \in \dot{\mathcal{E}}'], \tag{2.2}$$

where the integral is of Riemann-Stieltjes type and such that g is left-continuous at $s = a$ if a is not in E', and right-continuous at $s = 0$ if 0 is not in E'. For this unique g, $V_0^a(g) = \|\lambda\|$, where $V_0^a(g)$ denotes the Jordan variation of g over $[0, a]$.

Proof: Let $\mathcal{C}$ denote the space of continuous functions on $[0, a]$ with the usual norm. Since $\dot{\mathcal{E}}'$ is a closed vector subspace of $\mathcal{C}$, there exists by virtue of Theorem 2 of Banach[3] (p. 55) an extension λ_1 of λ, linear over $\mathcal{C}$, with $\|\lambda_1\| = \|\lambda\|$. According to a classical theorem, there exists a function g, with $g(0) = 0$, left-continuous on $[0, a)$, such that

$$\lambda_1(x) = \int_0^a x(s)\, dg(s) \qquad [x \in \mathcal{C}],$$

and with $V_0^a(g) = \|\lambda_1\|$. Moreover, g is left-continuous at $s = a$ if a is not in E'. Otherwise, let g_2 be defined on $[0, a]$ so that $g(s) = g_2(s)$ on $[0, a)$ and g_2 is left-continuous at $s = a$. Set

$$\lambda_2(x) = \int_0^a x(s)\, dg_2(s) \qquad [x \in \mathcal{C}]. \tag{2.3}$$

Then $\|\lambda_2\| = V_0^a(g_2) < V_0^a(g) = \|\lambda_1\| = \|\lambda\|$. But it is impossible that $\|\lambda_2\| < \|\lambda\|$. From equations (2.2) and (2.3) one sees that $\lambda_1(u) = \lambda_2(u)$ for $u \in \dot{\mathcal{E}}'$, since $u(a) = 0$. Hence $\|\lambda_2\| \geqq \|\lambda\|$. For similar reasons g is right-continuous at $s = 0$ if this point is not in E'.

If now g_1 and g_2 both satisfy the conditions on g of the lemma, by Lemma 2.1 the function $g_1 - g_2$ is constant on E' and, by continuity, on $[0, a]$ if $s = 0$ or $s = a$ is not in E'. Since $g_1(0) = g_2(0)$, it follows that $g_1 - g_2$ vanishes identically. The uniqueness of g is thus established, and the proof of the lemma is complete.

With E' and E'' intervals as in section 1, we designate by $\mathcal{C}'$ the space of functions continuous on $\bar{E}'$, and by $\mathcal{C}''$ the space of functions continuous on $\bar{E}''$, with

the usual norms. Let $\dot{\mathcal{E}}'$ be defined as in Lemma 2.2, and let $\dot{\mathcal{E}}''$ be similarly defined as the subspace of $\mathcal{C}''$ consisting of those functions which vanish outside of E''.

LEMMA 2.3. *A bounded* $\boldsymbol{C}$*-bilinear form* Λ *on* $\dot{\mathcal{E}}' \times \dot{\mathcal{E}}''$ *admits an extension* Λ_{12} *which is a* $\boldsymbol{C}$*-bilinear form on* $\mathcal{C}' \times \mathcal{C}''$ *for which* $\|\Lambda_{12}\| = \|\Lambda\|$.

Proof: For each $v \in \dot{\mathcal{E}}''$, $\Lambda(\cdot, v)$ defines a bounded $\boldsymbol{C}$-linear form λ_v on $\dot{\mathcal{E}}'$. Set $g_v = g_{\lambda_v}$ (cf. Lemma 2.2). Let $\Lambda_1(\cdot, v)$ denote the $\boldsymbol{C}$-measure on $\bar{E}'$ whose values are given by equation (2.2) for $u \in \mathcal{C}'$ when $g = g_v$. Since, for $v_1, v_2 \in \dot{\mathcal{E}}''$, $\lambda_{v_1} + \lambda_{v_2} = \lambda_{v_1+v_2}$ on $\dot{\mathcal{E}}'$, the uniqueness of g in Lemma 2.2 implies that $g_{v_1} + g_{v_2} = g_{v_1+v_2}$ and hence that $\Lambda_1(\cdot, v_1) + \Lambda_1(\cdot, v_2) = \Lambda_1(\cdot, v_1 + v_2)$. A similar argument shows that $\Lambda_1(\cdot, \alpha v) = \alpha\Lambda_1(\cdot, v)$ for $v \in \dot{\mathcal{E}}''$, $\alpha \in \boldsymbol{C}$. Thus Λ_1 is a $\boldsymbol{C}$-bilinear form on $\mathcal{C}' \times \dot{\mathcal{E}}''$. The relation

$$|\Lambda_1(u, v)| = \left|\int_0^a u(s)\, dg_v(s)\right| \leqq U(u)\, V_0^a(g_v) = U(u)\|\lambda_v\| \leqq U(u)\, U(v)\|\Lambda\|,$$

valid for $u \in \mathcal{C}'$, $v \in \dot{\mathcal{E}}''$, shows that Λ_1 is bounded with $\|\Lambda_1\| = \|\Lambda\|$. The form Λ_1, $\boldsymbol{C}$-bilinear on $\mathcal{C}' \times \dot{\mathcal{E}}''$ and bounded, similarly admits an extension Λ_{12}, $\boldsymbol{C}$-bilinear on $\mathcal{C}' \times \mathcal{C}''$, with $\|\Lambda_{12}\| = \|\Lambda_1\| = \|\Lambda\|$. This completes the proof of the lemma.

We state the principal theorem.

THEOREM 2.1. (i) *Corresponding to an arbitrary* $\boldsymbol{C}$*-bimeasure* Λ *on* $E' \times E''$ *there exists a regular function* k *such that, for* $(u, v) \in \mathcal{E}' \times \mathcal{E}''$,

$$\Lambda(u, v) = \int_{E'} u(s)\, d_s \int_{E''} v(t)\, d_t k(s, t), \tag{2.4}$$

where the integration of Riemann-Stieltjes type. The function k *may be chosen so as to have arbitrary null lines and is then uniquely determined by* Λ *and the choice of these lines. Furthermore,*

$$P_{\boldsymbol{C}}(k, E' \times E'') = \|\Lambda\|. \tag{2.5}$$

(ii) *Conversely, to each regular* k *there corresponds a unique* $\boldsymbol{C}$*-bimeasure* Λ *with values given by equation* (2.4) *and such that equation* (2.5) *holds.*

Proof of (*i*): Given an arbitrary point $(s_1, t_1) \in E' \times E''$, we introduce sequences E_n', E_n'', $n = 1, 2, \ldots$, of closed subintervals of E' and E'', respectively, with the following properties: (1) Setting $E_n' \times E_n'' = Q_n$, the point (s_1, t_1) is interior, relative to $E' \times E''$, to each Q_n. (2) For $m > n$, $Q_m \supset Q_n$. (3) $\operatorname{Union}_n Q_n = E' \times E''$. We shall denote by $F_n'[F_n'']$ the interval consisting of the interior points of $E_n'[E_n'']$ relative to $E'[E'']$. We note that $\operatorname{Union}_n F_n' \times F_n'' = E' \times E''$.

It will be convenient to suppose that a mapping $u \in \mathcal{E}_n'$ is extended in definition over E' by setting $u(s) = 0$ outside of E_n'. The mappings $v \in \mathcal{E}_n''$ are similarly supposed extended in definition over E''. The same convention is made concerning the spaces $\mathfrak{F}_n'$ and $\mathfrak{F}_n''$. The spaces $\dot{\mathcal{E}}_n'$ and $\dot{\mathcal{E}}_n''$ are closed vector subspaces of the spaces $\mathcal{E}_n'$ and $\mathcal{E}_n''$, respectively, subject to the convention just defined.

An arbitrary $\boldsymbol{C}$-bimeasure Λ then defines by restriction a bounded bilinear form Λ_n on $\dot{\mathcal{E}}_n' \times \dot{\mathcal{E}}_n''$. It is clear directly from their definitions that

$$\|\Lambda_n\| \leqq \|\Lambda\|, \qquad \lim_{n \uparrow \infty} \|\Lambda_n\| = \|\Lambda\|. \tag{2.6}$$

Since the proof of Lemma 2.3 evidently holds with the closed intervals $[0, a]$ and

$[0, b]$ replaced by arbitrary closed intervals, we apply Lemma 2.3, replacing $[0, a]$, $[0, b]$, E', and E'' in Lemma 2.3 by E_n', E_n'', F_n', and F_n'', respectively. With this replacement the spaces $\dot{\mathcal{E}}'$, $\dot{\mathcal{E}}''$, $\mathcal{C}'$, and $\mathcal{C}''$ of Lemma 2.3 become, respectively, $\dot{\mathcal{E}}_n'$, $\dot{\mathcal{E}}_n''$, $\mathcal{E}_n'$ and $\mathcal{E}_n''$. From Lemma 2.3 we have, then, an extension of Λ_n, denoted by Λ_n^{12}, which is a bounded bilinear form on $\mathcal{E}_n' \times \mathcal{E}_n''$, with $\|\Lambda_n^{12}\| = \|\Lambda_n\|$.

The application of Theorem 2.1 for closed intervals with E' and E'' replaced by E_n and E_n'', respectively, and Λ replaced by Λ_n^{12}, then gives a function k_n defined on $E_n' \times E_n''$, regular, with null lines $s = s_1$, $t = t_1$ on this rectangle, and such that

$$\Lambda_n^{12}(u, v) = \int_{E_n'} u(s)\, d_s \int_{E_n''} v(t)\, d_t k_n(s, t) \qquad [(u, v) \in \mathcal{E}_n' \times \mathcal{E}_n''], \tag{2.7}$$

$$\|\Lambda_n^{12}\| = P_{\mathcal{C}}(k_n, E_n' \times E_n'') \tag{2.8}$$

We next prove statement (α).

(α) *If $m > n$, then $k_m(s, t) = k_n(s, t)$ at each point (s, t) of $F_n' \times F_n''$.*

Observe, first, that $\mathfrak{F}_n' \subset \dot{\mathcal{E}}_n' \subset \dot{\mathcal{E}}_m'$ and $\mathfrak{F}_n'' \subset \dot{\mathcal{E}}_n'' \subset \dot{\mathcal{E}}_m''$. Hence, for $(u, v) \in \mathfrak{F}_n' \times \mathfrak{F}_n''$, by equation (2.7),

$$\Lambda(u, v) = \Lambda_n^{12}(u, v) = \int_{E_n'} u(s)\, d_s \int_{E_n''} v(t)\, d_t k_n(s, t),$$

$$\Lambda(u, v) = \Lambda_m^{12}(u, v) = \int_{E_n'} u(s)\, d_s \int_{E_n''} v(t)\, d_t k_m(s, t).$$

Hence

$$\int_{F_n'} u(s)\, d_s \int_{F_n''} v(t)\, d_t[k_n(s, t) - k_m(s, t)] = 0 \tag{2.9}$$

for all $(u, v) \in \mathfrak{F}_n' \times \mathfrak{F}_n''$, noting that for these functions the range of integration may be written either as E_n' or F_n' $[E_n''$ or $F_n'']$. Since for fixed $v \in \mathfrak{F}_n''$ the integral $J_v(s) = \int_{E_n'} v(t)\, d_t[k_n(s, t) - k_m(s, t)]$ is left-continuous except at most at the right end point of F_n' (see a preceding paper,[1] Lemma 9.4) and of bounded Jordan variation, equation (2.9), taken with Lemma 2.1, implies that, for $s \in F_n'$, $v \in \mathfrak{F}_n''$, $J_v(s) = J_v(s_1)$, that is,

$$\int_{E_n''} v(t)\, d_t[k_n(s, t) - k_m(s, t)] = \int_{F_n''} v(t)\, d_t[k_n(s_1, t) - k_m(s_1, t)].$$

Since $k_n(s_1, \cdot) \equiv k_m(s_1, \cdot) = 0$ on F_n'', it follows that

$$\int_{F_n''} v(t)\, d_t[k_n(s, t) - k_m(s, t)] = 0 \qquad [s \in F_n', v \in \mathfrak{F}_n''].$$

A second application of Lemma 2.1, and the fact that $k_m(\cdot, t_1) \equiv k_n(\cdot, t_1) \equiv 0$ on F_n', then shows that

$$k_n(s, t) - k_m(s, t) = k_n(s, t_1) - k_m(s, t_1) = 0 \qquad [(s, t) \in F_n' \times F_n''].$$

This completes the proof of (α).

Since every point of $E' \times E''$ is in $F_n' \times F_n''$ for some n, we can uniquely define $k(s, t)$ for each point of $E' \times E''$ as $k_n(s, t)$ for such an n.

Proof of equation (2.4): To see that equation (2.4) holds with this choice of k, it is only necessary to observe that if $(u, v) \in \mathcal{E}' \times \mathcal{E}''$, then, for some n, $(u, v) \in \dot{\mathcal{E}}_n' \times \dot{\mathcal{E}}_n''$, and, for such an n,

$$\Lambda(u, v) = \Lambda_n(u, v) = \Lambda_n^{12}(u, v) = \int_{E_n'} u(s)\, d_s \int_{E_n''} v(t)\, d_t k_n(s, t) \tag{2.10}$$

$$= \int_{F_n'} u(s)\, d_s \int_{F_n''} v(t)\, d_t k_n(s, t) = \int_{E'} u(s)\, d_s \int_{E''} v(t)\, d_t k(s, t).$$

We have used the fact that u and v vanish on $E' - F_n'$ and $E'' - F_n''$, respec-

tively, and that $k_n = k$ on $F_n' \times F_n''$. The function k clearly has null lines, $s = s_1$ and $t = t_1$, and the uniqueness of a k with these properties follows as in the proof of statement (α).

Proof of equation (2.5): We observe that the theorem for closed intervals gives

$$\|\Lambda_n^{12}\| = P_{\mathbf{C}}(k_n, Q_n). \tag{2.11}$$

Since every Q_n is contained in the interior, relative to $E' \times E''$, of some Q_m for $m > n$, it follows that, for such an m, $k_m | Q_n = k | Q_n$. Hence

$$\|\Lambda_m^{12}\| = P_{\mathbf{C}}(k_m, Q_m) \geqq P_{\mathbf{C}}(k_m, Q_n) = P_{\mathbf{C}}(k, Q_n). \tag{2.12}$$

From equation (2.12), from the relation $\|\Lambda_m^{12}\| = \|\Lambda_m\|$, and from relation (2.6), we find that

$$P_{\mathbf{C}}(k, Q_n) \leqq \|\Lambda\|, \tag{2.13}$$

and hence

$$P_{\mathbf{C}}(k, E' \times E'') \leqq \|\Lambda\|. \tag{2.14}$$

Moreover, for an arbitrary $(u, v) \in \mathcal{E}' \times \mathcal{E}''$ for which $U(x)\ U(y) > 0$, with n chosen as in equation (2.10),

$$\Lambda(u, v) = \int_{E_n'} u(s)\, d_s \int_{E_n''} v(t)\, d_t k(s, t) \leqq U(u)\ U(v)\ P_{\mathbf{C}}(k, Q_n),$$

using Theorem 2.1 for closed intervals. It follows that

$$\frac{\Lambda(u, v)}{U(u)\ U(v)} \leqq P_{\mathbf{C}}(k, Q_n) \leqq P_{\mathbf{C}}(k, E' \times E'').$$

Hence

$$\|\Lambda\| \leqq P_{\mathbf{C}}(k, E' \times E''). \tag{2.15}$$

Inequalities (2.14) and (2.15) establish equation (2.5), and this completes the proof of (i).

Proof of (ii): For a regular k satisfying the conditions of (ii), $P_{\mathbf{C}}(k, Q_n) < \infty$ for each n. For $(u, v) \in \dot{\mathcal{E}}_n' \times \dot{\mathcal{E}}_n''$, granting Theorem 2.1 for closed intervals, the existence of the integral $\Lambda(u, v)$ in equation (2.4) follows, as well as the relation

$$|\Lambda(u, v)| \leqq U(u)\ U(v)\ P_{\mathbf{C}}(k, Q_n) \qquad [(u, v) \in \dot{\mathcal{E}}_n' \times \dot{\mathcal{E}}_n''] \tag{2.16}$$

Hence $\Lambda(u, v)$ is a $\mathbf{C}$-bimeasure on $E' \times E''$, and

$$\|\Lambda\| \leqq \sup_n P_{\mathbf{C}}(k, Q_n) = P_{\mathbf{C}}(k, E' \times E'').$$

Let k' be defined for $(s, t) \in E' \times E''$ by setting

$$k'(s, t) = k(s, t) - k(s_1, t) - k(s, t_1) + k(s_1, t_1).$$

Applying (i) to $\Lambda(u, v)$, one sees that k' satisfies the conditions on the function k affirmed to exist in (i), and hence, from the uniqueness of such a function and from equation (2.5),

$$P_{\mathbf{C}}(k', E' \times E'') = \|\Lambda\|.$$

Since $P_{\mathbf{C}}(k', E' \times E'') = P_{\mathbf{C}}(k, E' \times E'')$ in accordance with the definition of the

Fréchet variation,

$$P_C(k, E' \times E'') = \|\Lambda\|.$$

Thus the proof of (ii) and of the theorem is complete.

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ON THE KRONECKER PRODUCT OF IRREDUCIBLE REPRESENTATIONS OF THE SYMMETRIC GROUP

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We have treated in a recent note in these Proceedings[1] the problem of analyzing the Kronecker product $\Gamma(n - p', (\mu')) \times \Gamma(n - p, (\mu))$ of two irreducible representations of S_n, the symmetric group on n symbols, into its irreducible components and have given rules which furnish this analysis when p', which we take to be $\leq p$, is not greater than 3. While this may be sufficient for physical applications, it is not satisfactory, due to its incompleteness, from the mathematical point of view. The object of the present note is to furnish the analysis of $\Gamma(n - p',(\mu')) \times \Gamma(n - p, (\mu))$ for all irreducible representations of S_n, i.e., for all partitions (μ) and (μ') of p and p', respectively.

We have pointed out in the note referred to that the terms of greatest depth, namely, $p + p'$ in $\Gamma(n - p', (\mu')) \times \Gamma(n - p, (\mu))$, which we write simply as $(\mu') \times (\mu)$, are furnished by the product $\{\mu'\}\{\mu\}$ of the two irreducible representations $\{\mu\}$ and $\{\mu'\}$, of degrees p and p', respectively, of the n-dimensional linear group. For example, the terms of depth $p + 1$ in $(1) \times (\mu)$ are furnished by $\{\mu\} S_1$, where we denote $\{1\}$ by S_1. Similarly, the terms of depth $p + 2$ in $(S_2) \times (\mu)$, where $(S_2) = (2) - (1^2)$, are furnished by $\{\mu\}S_2$; the terms of depth $p + 3$ in $(S_3) \times (\mu)$, where $(S_3) = (3) - (21) + (1^3)$, are furnished by $\{\mu\}S_3$; and so on. If $(\mu) = (\mu_1, \ldots, \mu_j)$ has j nonzero parts, the various products $\{\mu\}S_1$, $\{\mu\}S_2$, $\{\mu\}S_3$, and so on, are furnished by the following formulas:

$$\{\mu\}S_1 = \{\mu_1 + 1, \mu_2, \ldots, \mu_j\} + \{\mu_1, \mu_2 + 1, \mu_3, \ldots, \mu_j\} + \ldots + \{\mu_1, \ldots, \mu_j + 1\} + \{\mu_1, \ldots, \mu_j, 1\};$$

$$\{\mu\}S_2 = \{\mu_1 + 2, \mu_2 \ldots, \mu_j\} + \{\mu_1, \mu_2 + 2, \mu_3, \ldots, \mu_j\} + \ldots + \{\mu_1, \ldots, \mu_j + 2\} + \{\mu_1, \ldots, \mu_j, 2\} - \{\mu_1, \ldots, \mu_j, 1^2\};$$

$$\{\mu\}S_3 = \{\mu_1 + 3, \mu_2, \ldots, \mu_j\} + \{\mu_1, \mu_2 + 3, \mu_3, \ldots, \mu_j\} + \ldots + \{\mu_1, \ldots, \mu_j + 3\} + \{\mu_1, \ldots, \mu_j, 3\} - \{\mu_1, \ldots, \mu_j, 21\} + \{\mu_1, \ldots, \mu_j, 1^3\};$$

and so on. The complete analysis of (1) $\times$ (μ) is furnished by $\{\mu\}S_1 + \{\mu/S_1\}\ (S_1 + 1)$, where $\{\mu/S_1\} = \{\mu_1 - 1, \mu_2, \ldots, \mu_j\} + \ldots + \{\mu_1, \ldots, \mu_{j-1}, \mu_j - 1\}$, and we indicate this result by the symbol

$$(1) \times (\mu) \rightarrow \{\mu\}S_1 + \{\mu/S_1\}\ (S_1 + 1).$$

Similarly,

$$(S_2) \times (\mu) \rightarrow \{\mu\}S_2 + \{\mu/S_2\}\ (S_2 + 1),$$
$$(S_3) \times (\mu) \rightarrow \{\mu\}S_3 + \{\mu/S_3\}\ (S_3 + 1),$$

and so on, where

$$\{\mu/S_2\} = \{\mu_1 - 2, \mu_2, \ldots, \mu_j\} + \ldots + \{\mu_1, \ldots, \mu_{j-1}, \mu_j - 2\},$$
$$\{\mu/S_3\} = \{\mu_1 - 3, \mu_2, \ldots, \mu_j\} + \ldots + \{\mu_1, \ldots, \mu_{j-1}, \mu_j - 3\},$$

and so on.

After these preliminaries we are ready to write down the formula which furnishes the analysis of (μ') $\times$ (μ); it is

$$(\mu') \times (\mu) \rightarrow \{\mu'\}\{\mu\} + [\{\mu'/S_1\}\{\mu/S_1\}\ (S_1 + 1)] + \frac{1}{2}[\{\mu'/S_1^2\}\{\mu/S_1^2\}\ (S_1 + 1)^2$$
$$+ \{\mu'/S_2\}\{\mu/S_2\}\ (S_2 + 1)] + \frac{1}{6}[\{\mu'/S_1^3\}\{\mu/S_1^3\}\ (S_1 + 1)^3 +$$
$$3\{\mu'/S_1S_2\}\{\mu/S_1S_2\}\ (S_1 + 1)\ (S_2 + 1) + 2\{\mu'/S_3\}\{\mu/S_3\}\ (S_3 + 1)] + \ldots,$$

where the coefficients in each [. . .] are the numbers of elements in the various classes of the symmetric groups on 1, 2, 3, . . . , symbols. Thus the next term in the unfinished expression just written down is

$$\frac{1}{24}[\{\mu'/S_1^4\}\{\mu/S_1^4\}\ (S_1 + 1)^4 + 6\{\mu'/S_1^2S_2\}\{\mu/S_1^2S_2\}\ (S_1 + 1)^2(S_2 + 1) +$$
$$8\{\mu'/S_1S_3\}\{\mu/S_1S_3\}\ (S_1 + 1)\ (S_3 + 1) + 3\{\mu'/S_2^2\}\{\mu/S_2^2\}\ (S_2 + 1)^2 +$$
$$6\{\mu'/S_4\}\{\mu/S_4\}\ (S_4 + 1)].$$

If $\Gamma(n - p', (\mu'))$ is of depth 2, so that (μ') is a partition of 2, all the terms after $\frac{1}{2}[\{\mu'/S_1^2\}\{\mu/S_1^2\}\ (S_1 + 1)^2 + \{\mu'/S_2\}\{\mu/S_2\}\ (S_2 + 1)]$ vanish, and (μ') $\times$ (μ) is furnished by the formula

$$(\mu') \times (\mu) \rightarrow \{\mu'\}\{\mu\} + [\{\mu'/S_1\}\{\mu/S_1\}\ (S_1 + 1)] +$$
$$\frac{1}{2}[\{\mu'/S_1^2\}\{\mu/S_1^2\}\ (S_1 + 1)^2 + \{\mu'/S_2\}\{\mu/S_2\}\ (S_2 + 1)].$$

For example,

$$(2) \times (\mu) \rightarrow \{2\}\{\mu\} + \{\mu/S_1\}\ (S_1^2 + S_1) + \frac{1}{2}[\{\mu/S_1^2\}\ (S_1 + 1)^2 +$$
$$\{\mu/S_2\}\ (S_2 + 1)],$$

where $S_1^2 = \{2\} + \{1^2\}$, $S_1 = \{1\}$, $S_2 = \{2\} - \{1^2\}$. If $\Gamma(n - p', (\mu'))$ is of depth

[3]/we have merely to add the term $\frac{1}{6}$ $[\{\mu'/S_1^3\}$ $\{\mu/S_1^3\}$ $(S_1 + 1)^3 + 3\{\mu'/S_1S_2\}$ $\{\mu/S_1S_2\}$ $(S_1 + 1)$ $(S_2 + 1) + 2\{\mu'/S_3\}$ $\{\mu/S_3\}$ $(S_3 + 1)]$ to the expression which furnished $(\mu') \times (\mu)$ when $\Gamma(n - p', (\mu'))$ was of depth 2, and so on. It follows from the general formula just given that

1. The terms of depth $p + p'$ in $(\mu') \times (\mu)$ are furnished by $\{\mu'\}$ $\{\mu\}$;
2. The terms of depth $p + p' - 1$ in $(\mu') \times (\mu)$ are furnished by $\{\mu'/S_1\}$ $\{\mu/S_1\}S_1$;
3. The terms of depth $p + p' - 2$ in $(\mu') \times (\mu)$ are furnished by

$$\{\mu'/S_1\}\ \{\mu/S_1\} + \frac{1}{2}\ [\{\mu'/S_1^2\}\ \{\mu/S_1^2\}\ S_1^2 + \{\mu'/S_2\}\ \{\mu/S_2\}S_2];$$

4. The terms of depth $p + p' - 3$ in $(\mu') \times (\mu)$ are furnished by

$$\{\mu'/S_1^2\}\ \{\mu/S_1^2\}S_1 + \frac{1}{6}\ [\{\mu'/S_1^3\}\ \{\mu/S_1^3\}S_1^3 + 3\{\mu'/S_1S_2\}\ \{\mu/S_1S_2\}S_1S_2 + 2\{\mu'/S_3\}\ \{\mu/S_3\}S_3];$$

5. The terms of depth $p + p' - 4$ in $(\mu') \times (\mu)$ are furnished by

$$\frac{1}{2}\ [\{\mu'/S_1^2\}\ \{\mu/S_1^2\} + \{\mu'/S_2\}\ \{\mu/S_2\}] + \frac{1}{2}[\{\mu'/S_1^3\}\ \{\mu/S_1^3\}S_1^2 +$$

$$\{\mu'/S_1S_2\}\ \{\mu/S_1S_2\}S_2] + \frac{1}{24}\ [\{\mu'/S_1^4\}\ \{\mu/S_1^4\}S_1^4 + 6\{\mu'/S_1^2S_2\}\ \{\mu/S_1^2S_2\}S_1^2S_2 +$$

$$8\{\mu'/S_1S_3\}\ \{\mu/S_1S_3\}S_1S_3 + 3\{\mu'/S_2^2\}\ \{\mu/S_2^2\}S_2^2 + 6\{\mu'/S_4\}\ \{\mu/S_4\}S_4];$$

6. The terms of depth $p + p' - 5$ in $(\mu') \times (\mu)$ are furnished by

$$\frac{1}{2}\ [\{\mu'/S_1^3\}\ \{\mu/S_1^3\} + \{\mu'/S_1S_2\}\ \{\mu/S_1S_2\}]S_1 + \frac{1}{6}\ [\{\mu'/S_1^4\}\ \{\mu/S_1^4\}S_1^3 +$$

$$3\{\mu'/S_1^2S_2\}\ \{\mu/S_1^2S_2\}S_1S_2 + 2\{\mu'/S_1S_3\}\ \{\mu/S_1S_3\}S_3] + \frac{1}{120}\ [\{\mu'/S_1^5\}\ \{\mu/S_1^5\}\ S_1^5 +$$

$$10\{\mu'/S_1^3S_2\}\ \{\mu/S_1^3S_2\}S_1^3S_2 + 20\{\mu'/S_1^2S_3\}\ \{\mu/S_1^2S_3\}S_1^2S_3 +$$

$$15\{\mu'/S_1S_2^2\}\ \{\mu/S_1S_2^2\}S_1S_2^2 + 30\{\mu'/S_1S_4\}\ \{\mu/S_1S_4\}S_1S_4 + 20\{\mu'/S_2S_3\}$$

$$\{\mu/S_2S_3\}\ S_2S_3 + 24\{\mu'/S_5\}\ \{\mu/S_5\}S_5];$$

and so on. Similarly:

1′. The terms of depth $p - p'$ in $(\mu') \times (\mu)$ are furnished by $\{\mu/\mu'\}$;

2′. The terms of depth $p - p' + 1$ in $(\mu') \times (\mu)$ are furnished by $(\{\mu/S_1\} \div \{\mu'/S_1\})S_1$;

3′. The terms of depth $p - p' + 2$ in $(\mu') \times (\mu)$ are furnished by

$$(\{\mu\} \div \{\mu'/S_1\})\ S_1 + \frac{1}{2}\ [(\{\mu/S_1^2\} \div \{\mu'/S_1^2\})S_1^2 + (\{\mu/S_2\} \div \{\mu'/S_2\})S_2];$$

4′. The terms of depth $p - p' + 3$ in $(\mu') \times (\mu)$ are furnished by

$$(\{\mu/S_1\} \div \{\mu'/S_1^2\})S_1^2 + \frac{1}{6}\ [(\{\mu/S_1^3\} \div \{\mu'/S_1^3\})S_1^3 +$$

$$3(\{\mu/S_1S_2\} \div \{\mu'/S_1S_2\})S_1S_2 + 2(\{\mu/S_3\} \div \{\mu'/S_3\})S_3];$$

5′. The terms of depth $p - p' + 4$ in $(\mu') \times (\mu)$ are furnished by

$$\frac{1}{2}[(\{\mu/S_1\} \div \{\mu'/S_1^3\})S_1^2 + (\{\mu\} \div \{\mu'/S_2\})S_2] +$$
$$\frac{1}{2}[(\{\mu/S_1^2\} \div \{\mu'/S_1^3\})S_1^2 + (\{\mu/S_2\} \div \{\mu'/S_1S_2\})S_2]S_1 +$$
$$\frac{1}{24}[(\{\mu/S_1^4\} \div \{\mu'/S_1^4\})S_1^4 + 6(\{\mu/S_1^2S_2\} \div \{\mu'/S_1^2S_2\})S_1^2S_2 +$$
$$8(\{\mu/S_1S_3\} \div \{\mu'/S_1S_3\})S_1S_3 + 3(\{\mu/S_2^2\} \div \{\mu'/S_2^2\})S_2^2 +$$
$$6(\{\mu/S_4\} \div \{\mu'/S_4\})S_4];$$

6′. The terms of depth $p - p' + 5$ in $(\mu') \times (\mu)$ are furnished by

$$\frac{1}{2}[(\{\mu/S_1\} \div \{\mu'/S_1^3\})S_1^2 + (\{\mu/S_1\} \div \{\mu'/S_1S_2\})S_2]S_1 +$$
$$\frac{1}{6}[(\{\mu/S_1^3\} \div \{\mu'/S_1^4\})S_1^3 + 3(\{\mu/S_1S_2\} \div \{\mu'/S_1^2S_2\})S_1S_2 +$$
$$2(\{\mu/S_3\} \div \{\mu'/S_1S_3\})S_3]S_1 + \frac{1}{120}[(\{\mu/S_1^5\} \div \{\mu'/S_1^5\})S_1^5 +$$
$$10(\{\mu/S_1^3S_2\} \div \{\mu'/S_1^3S_2\})S_1^3S_2 + 20(\{\mu/S_1^2S_3\} \div \{\mu'/S_1^2S_3\})S_1^2S_3 +$$
$$15(\{\mu/S_1S_2^2\} \div \{\mu'/S_1S_2^2\})S_1S_2^2 + 30(\{\mu/S_1S_4\} \div \{\mu'/S_1S_4\})S_1S_4 +$$
$$20(\{\mu/S_2S_3\} \div \{\mu'/S_2S_3\})S_2S_3 + 24(\{\mu/S_5\} \div \{\mu'/S_5\})S_5];$$

and so on. For example, the terms of depth 10 in (5) × (5) are (10) + (91) + (82) + (73) + (64) + (5^2); the terms of depth 9 are (9) + 2(81) + 2(72) + (71^2) + 2(63) + (621) + 2(54) + (531) + (4^21); the terms of depth 8 are 2(8) + 3(71) + 4(62) + (61^2) + 4(53) + 2(521) + 2(4^2) + 2(431) + (42^2) + (3^22); the terms of depth 7 are 2(7) + 4(61) + 5(52) + 2(51^2) + 4(43) + 3(421) + 2(3^21) + (32^2); the terms of depth 6 are 3(6) + 5(51) + 5(42) + 2(41^2) + 2(3^2) + 2(321) + (2^3); the terms of depth 5 are 3(5) + 4(41) + 3(32) + 2(31^2) + (2^21); the terms of depth 4 are 3(4) + 3(31) + 2(2^2) + (21^2); the terms of depth 3 are 2(3) + 2(21) + (1^3); the terms of depth 2 are 2(2) + (1^2); the term of depth 1 is (1); and the term of depth 0 is (0).

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[1] F. D. Murnaghan, "On the Irreducible Representations of the Symmetric Group," these PROCEEDINGS, **41**, 1096–1103, 1955.

*ON GENERALIZED TCHEBYCHEFF POLYNOMIALS**

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1. If E is a bounded finite or infinite closed point set of the z-plane, the Tchebycheff polynomial $T_n(z)$ of degree n for E is defined as that polynomial of the form

$$T_n(z) \equiv z^n + a_1 z^{n-1} + \ldots + a_n$$

for which the norm

$$\max [|T_n(z)|, \quad z \text{ on } E]$$

is least. It can be shown that $T_n(z)$ exists and, if E consists of more than $n - 1$ points, is unique.

Tchebycheff polynomials arise naturally from the following problem in approximation: *Given a function $f(z)$ defined on E, find a polynomial of the form*

$$P(z) \equiv p_0 z^{n-1} + p_1 z^{n-2} + \ldots + p_{n-1}$$

such that the norm

$$\max [|f(z) - P(z)|, \quad z \text{ on } E] \tag{1}$$

is least. The extremal approximating polynomial $P(z)$ is $z^n - T_n(z)$ if $f(z) \equiv z^n$.

2. The object of the present note is to set forth some results on the following more general problem: *Given a polynomial $f(z) \equiv z^n + A_1 z^{n-1} + \ldots + A_n$, find a polynomial of the form*

$$P(z) \equiv p_0 z^{n-s-1} + p_1 z^{n-s-2} + \ldots + p_{n-s-1} \tag{2}$$

such that the norm (1) *is least.* This problem gives rise to a *generalized Tchebycheff polynomial $T_n^s(z)$ of the form*

$$T_n^s(z) \equiv z^n + A_1 z^{n-1} + \ldots + A_s z^{n-s} + a_{s+1} z^{n-s-1} + \ldots + a_n, \tag{3}$$

where $A_1, \ldots, A_s$ are prescribed and for which the norm

$$\max [|T_n^s(z)|, \quad z \text{ on } E] \tag{4}$$

is least. The extremal approximating polynomial is then $P(z) = f(z) - T_n^s(z)$. It can be shown that $T_n^s(z)$ exists and, if E contains more than $n - s - 1$ points, is unique. If E contains precisely $n - s$ points, $T_n^s(z)$ must be chosen to vanish in all points of E. Henceforth we assume that E contains more than $n - s - 1$ points.

Some time ago the senior author suggested to the junior author the study of the properties of the $T_n^s(z)$ as a promising field for research, especially with reference to a possible generalization of Fejér's principle (see sec. 6 below). He indicated in particular the proof of the following illuminating and suggestive result: *If the point set E contains at least $n - 1$ points and lies in the interval $a \leq x \leq b$, and if the given number A_1 is real, then the extremal polynomial $T_n^1(z) \equiv z^n + A_1 z^{n-1} + a_2 z^{n-2} + \ldots + a_n$ has zeros all of which lie on the intervals $a \leq x \leq b$, and $(-A_1 -$*

$(n-1)b) \leq x \leq (-A_1 - (n-1)a)$. *If these two intervals are disjoint, they contain, respectively,* $n-1$ *and* 1 *zeros of* $T_n^1(z)$. The proof relies on the fact, first, that all zeros of $T_n^1(z)$ must be real, for otherwise $|T_n^1(z)|$ could be diminished simultaneously for all points z of E by replacing all the zeros by their projections on the axis of reals, and, second, that if two real zeros of $T_n^1(z)$ are outside $a \leq x \leq b$, they can be moved without changing their center of gravity until one zero lies in $a \leq x \leq b$, also in such a manner that $|T_n^1(z)|$ is diminished simultaneously for all points z of E. The conclusion is valid not only for $T_n^1(z)$ but also for infrapolynomials (sec. 5 below). Under these conditions, with $n > 1$, any two zeros of $T_n^1(z)$ are separated by at least one point of E; otherwise, these two zeros can be moved farther away from each other without changing their center of gravity, so as to decrease the norm of $T_n^1(z)$ on E.

3. The computation of Tchebycheff polynomials, classical and generalized alike, has been carried out only in very special cases. For E the interval $-1 \leq x \leq 1$, $s = 1$, and A_1 real, $T_n^1(z)$ is known explicitly (Zolotarev[1]). For E the same interval, $s = 2$, $A_1 = 0$, and A_2 real, $T_n^2(z)$ is known (Achyeser[2]). Fekete and von Neumann[3] have computed $T_n^0(z)$ for a set E which consists of exactly $n+1$ points $z_0, z_1, \ldots, z_n$, namely,

$$T_n^0(z) \equiv \frac{\sum_0^n (1/|g'(z_i)|) \cdot g(z)/(z - z_i)}{\sum_0^n (1/|g'(z_i)|)}, \qquad g(z) \equiv \prod_0^n (z - z_i). \tag{5}$$

4. The results which follow are due to the junior author, subsequent to the suggestions already detailed.

The polynomial $T_n^s(z)$ can also be computed for a set E which consists of exactly $n - s + 1$ points. Denoting them by $z_0, z_1, \ldots, z_{n-s}$ and setting $g(z) \equiv \prod_0^{n-s} (z - z_i)$, we can represent the fixed part of $T_n^s(z)$ as given in relation (3),

$$A(z) \equiv z^n + A_1 z^{n-1} + \ldots + A_s z^{n-s},$$

at the points of E by a polynomial

$$A_1(z) \equiv \sum_0^{n-s} \frac{A(z_i)\, g(z)}{g'(z_i)\,(z - z_i)} = B_0 z^{n-s} + \ldots,$$

and we have $A(z_i) = A_1(z_i)$ for $i = 0, 1, \ldots, n-s$. Denoting $Q_{n-s}(z) \equiv A_1(z) + a_{s+1} z^{n-s-1} + \ldots + a_n \equiv B_0 z^{n-s} + \ldots$ and assuming that it minimizes the norm $\max [|Q_{n-s}(z)|, z \text{ on } E]$ as compared to any other polynomial of the same degree and with the same leading coefficient, we obtain $Q_{n-s}(z) = B_0\, T_{n-s}^0(z)$, whence $T_n^s(z) = A(z) + B_0 T_{n-s}^0(z) - A_1(z)$, a relation which holds true not merely for the Tchebycheff norm (4) but also for other norms, such as least pth powers ($p > 0$) with weight functions. For the norm (4) the polynomial $T_{n-s}^0(z)$ is computed in relation (5), and therefore $T_n^s(z)$ is given explicitly by

$$T_n^s(z) = A(z) + \sum_{i=0}^{n-s} \frac{\left(\sum_{k=0}^{n-s} (A(z_k)/g'(z_k))\right) T_{n-s}^0(z) - A(z_i)}{g'(z_i)} \cdot \frac{g(z)}{z - z_i}. \tag{6}$$

5. The fact that Tchebycheff polynomials—even the classical ones—are so difficult to compute attaches more significance to problems of their structure and to theorems about the location of their zeros. Thus far, all these properties have been established for a wider class of polynomials, namely, the so-called "infra-polynomials." It is the main purpose of this note to generalize such properties for the $T_n^s(z)$ as defined in section 2 and for the wider class of infra-(n, s)-polynomials defined as follows: *Given a point set E as in section 2, positive integers n and s $(s < n)$, complex numbers $A_1, A_2, \ldots, A_s$, we define as a corresponding infra-(n, s)-polynomial the polynomial*

$$I_n^s(z) \equiv z^n + A_1 z^{n-1} + \ldots + A_s z^{n-s} + a_{s+1} z^{n-s-1} + \ldots + a_n,$$

if there is no "underpolynomial" on E, that is, if there is no other polynomial

$$p_n^s(z) = z^n + A_1 z^{n-1} + \ldots + A_s z^{n-s} + c_{s+1} z^{n-s-1} + \ldots + c_n$$

having the same $s + 1$ leading coefficients and such that $|p_n^s(z)| < |I_n^s(z)|$ for z on E, except, perhaps, for common zeros, where $p_n^s(z) = I_n^s(z) = 0$. The ordinary infra-polynomials correspond to the case $s = 0$, when only the first leading coefficient 1 is given.

Every generalized Tchebycheff polynomial is a corresponding infra-(n, s)-polynomial. In fact, if instead of the norm (4) we use any other monotonic norm in the sense of Fejér,[4] that is, one which decreases if a polynomial is replaced by any one of its underpolynomials, the resulting extremal polynomial will be an infra-(n, s)-polynomial. For connections between infrapolynomials and derivatives see Fekete[5] and Motzkin and Walsh.[6]

6. A well-known theorem of Fejér[4] states that all the zeros of an infrapolynomial (and hence also of $T_n(z)$) lie in the convex hull of E. This is a geometric result and can be generalized for the location of zeros of $I_n^s(z)$ (and hence also for $T_n^s(z)$) as follows (stated by Zedek[7] for the $T_n^s(z)$):

THEOREM 1. *Under the notations and hypotheses of section 5, let $z_0^*, z_1^*, \ldots, z_s^*$ be a group of $s + 1$ zeros of $I_n^s(z)$,* and let $\phi_0, \phi_1, \ldots, \phi_s$ *be the angles subtended by E at these zeros, respectively. Then*

$$\phi_0 + \phi_1 \ldots + \phi_s \geq \pi. \tag{7}$$

Factor $I_n^s(z)$ into $q_{n-s-1}(z) r_{s+1}(z)$, where $r_{s+1}(z) = \prod_0^s (z - z_i^*)$ and $q_{n-s-1}(z)$ is the appropriate polynomial of degree $n - s - 1$. Let ζ_1 and ζ_2 be two arbitrary points in E. Then

$$\arg r_{s+1}(\zeta_1) - \arg r_{s+1}(\zeta_2) = \sum_0^s \{\arg (\zeta_1 - z_i^*) - \arg (\zeta_2 - z_i^*)\};$$

but $|\arg (\zeta_1 - z_i^*) - \arg (\zeta_2 - z_i^*)|$ is the angle subtended at z_i^* by the segment connecting ζ_1 and ζ_2 and is certainly not larger than ϕ_i. Hence $|\arg r_{s+1}(\zeta_1) - \arg r_{s+1}(\zeta_2)| \leq \phi_0 + \phi_1 + \ldots + \phi_s$. Now assume by way of contradiction that $\phi_0 + \phi_1 + \ldots + \phi_s < \pi$, whence $|\arg r_{s+1}(\zeta_1) - \arg r_{s+1}(\zeta_2)| < \pi$. This can be expressed geometrically by saying that the image of E under the transformation $w = r_{s+1}(z)$ is a closed set situated inside an angle smaller than π whose vertex is $w = 0$. We may assume that the origin itself is not included in the image, because

that would mean that one of the zeros z_i^* is in E or that, by convention, $\phi_i = 2\pi$, and hence relation (7) will be trivially true. There exists, then, a constant c such that $|r_{s+1}(z) + c| < |r_{s+1}(z)|$ when z lies on E. We observe that the polynomial

$$h_n(z) = q_{n-s-1}(z)\,[r_{s+1}(z) + c] = z^n + A_1 z^{n-1} + \ldots + A_s z^{n-s} + \ldots$$

has the same $s + 1$ first coefficients as $I_n^s(z)$ and yet $|h_n(z)| < |I_n^s(z)|$ on E, which contradicts the very definition of $I_n^s(z)$.

7. The above result is a true generalization of Fejér's theorem. Indeed, if $s = 0$, relation (7) becomes $\phi_0 \geq \pi$, which means that z_0^* is in the convex hull of E.

These results may be paraphrased geometrically by saying that, although some individual zeros may be far away from E (as $-A_1/n$ is the given center of gravity and is arbitrary), collectively they must be close to E, and a number of zeros corresponding to the degree of freedom of the problem must lie in an "inflated" convex hull of E.

By using relation (7) for all possible combinations of zeros of $I_n^s(z)$, we obtain a result proved by Fekete[8] subsequently to Zedek:[7] *at least* $n - s$ *of the angles* ϕ_i *are larger than* $\pi/(s + 1)$.

8. Before turning to the structure of $I_n^s(z)$, we emphasize that it is permissible to eliminate those zeros of our polynomials which coincide with points of E. Suppose $z_0^* \in E$. By writing

$$z^n + A_1 z^{n-1} + \ldots + A_s z^{n-s} + a_{s+1} z^{n-s-1} + \ldots + a_n = (z - z_0^*)\,(z^{n-1} + b_1 z^{n-2} + \ldots + b_{n-1}),$$

we obtain a set of equations, $b_1 - z_0^* = A_1$, $b_2 - z_0^* b_1 = A_2, \ldots, b_s - z_0^* b_{s-1} = A_s$, from which we may see that the first s coefficients in $z^{n-1} + b_1 z^{n-2} + \ldots + b_{n-2}$ are fixed, and the problem of studying $I_n^s(z)$ as an infra-(n, s)-polynomial on E is reduced to the problem of studying an infra-$(n - 1, s)$-polynomial on $E - z_0^*$.

We shall assume hereafter that $I_n^s(z)$ does not vanish on E.

9. Fekete proved[5] that for $I_n^0(z)$ the following orthogonality relations hold: *Let* E *be a point set as described in section* 1 *but consisting of at least* $n + 2$ *points. Let* $I_n^0(z)$ *be a corresponding infrapolynomial which differs from zero when* z *ranges over* E. *Then there are* $m + 1$ *points in* E, *say,* $z_0, z_1, \ldots, z_m$, *with* $n \leq m \leq 2n$ *and* $m + 1$ *positive constants, say,* $\lambda_0, \lambda_1, \ldots, \lambda_m$, *with* $\sum_0^m \lambda_\mu = 1$, *such that*

$$\sum_{\mu=0}^{m} \lambda_\mu \frac{z_\mu^{\alpha}}{I_n^0(z_\mu)} = 0 \qquad \text{for } \alpha = 0, 1, \ldots, n - 1, \tag{8}$$

and $I_n^0(z)$ *is an infrapolynomial also on the subset* $e = \{z_0, z_1, \ldots, z_m\}$ *of* E.

From here Fekete went on to prove the following interesting structure theorem:

$I_n^0(z)$ *admits the representation* $I_n^0(z) = \Lambda(z)/M(z)$, *where* $\Lambda(z)$ *and* $M(z)$ *are polynomials of the respective orders* m *and* $m - n$ *and with leading coefficients equal to* 1:

$$\Lambda(z) = \sum_{\mu=0}^{m} \lambda_\mu \frac{g(z)}{z - z_\mu}; \qquad M(z) = \sum_{\mu=0}^{m} \frac{\lambda_\mu}{I_n^0(z_\mu)} \frac{g(z)}{z - z_\mu}; \qquad g(z) = \prod_{\mu=0}^{m} (z - z_\mu). \tag{9}$$

10. The orthogonality relations were generalized by Fekete from a somewhat different point of view[8] and independently by the present authors; we shall merely restate them in the following form:

Let E be a point set as in section 1 but consisting of at least $n - s + 2$ points. Let $I_n^s(z) = z^n + A_1 z^{n-1} + \ldots + A_s z^{n-s} + a_{s+1} z^{n-s-1} + \ldots + a_n$ be a corresponding infra-(n, s)-polynomial which differs from zero when z ranges over E. Then there are $m + 1$ points in E, say, $z_0, z_1, \ldots, z_m$, with $n - s \leq m \leq 2(n - s)$ and $m + 1$ positive constants $\lambda_0, \lambda_1, \ldots, \lambda_m$ with $\sum_0^m \lambda_\mu = 1$, such that

$$\sum_{\mu=0}^{m} \lambda_\mu \frac{z_\mu^{\alpha}}{I_n^s(z_\mu)} = 0 \qquad \text{for } \alpha = 0, 1, \ldots, n - s - 1, \tag{10}$$

and $I_n^s(z)$ is an infra-(n, s)-polynomial also on the subset $e = \{z_0, z_1, \ldots, z_m\}$ of E.

It is now clear that the consideration of finite sets rather than of infinite sets is less of a restriction than it might appear at first glance.

11. We conclude this paper by proving a generalized structure theorem for infra-(n, s)-polynomials:

THEOREM 2. *Under the conditions of section 10, $I_n^s(z)$ admits the representation*

$$I_n^s(z) = \frac{p_s(z)\, \Lambda^*(z)}{M^*(z)}, \tag{11}$$

where $p_s(z)$ is any factor of $I_n^s(z)$ of degree s having as leading coefficient 1; $\Lambda^*(z)$, $M^*(z)$ are polynomials given by the formulas

$$\left.\begin{aligned} M^*(z) &= \sum_{\mu=0}^{m} \frac{\lambda_\mu z_\mu^{\gamma}}{I_n^s(z_\mu)} \frac{g(z)}{z - z_\mu}, \\ \Lambda^*(z) &= \sum_{\mu=0}^{m} \frac{\lambda_\mu z_\mu^{\gamma_0}}{p_s(z_\mu)} \frac{g(z)}{z - z_\mu}, \\ g(z) &= \prod_{\mu=0}^{m} (z - z_\mu); \end{aligned}\right\} \tag{12}$$

and γ_0 satisfies $0 \leq \gamma_0 \leq s$.

To prove Theorem 2, consider the sums

$$\sum_{\mu=0}^{m} \lambda_\mu \frac{z_\mu^{\alpha}}{I_n^s(z)}$$

for $\alpha = n - s, \ldots, n$. Let α_0 be the first index α for which the above sum is different from zero. Such an index exists, because if all these sums were equal to zero, we would get the following contradiction:

$$0 = \sum_{\alpha=0}^{n} b_\alpha \sum_{\mu=0}^{m} \frac{\lambda_\mu z_\mu^{\alpha}}{I_n^s(z_\mu)} = \sum_{\mu=0}^{m} \frac{\lambda_\mu \sum_{\alpha=0}^{n} b_\alpha z_\mu^{\alpha}}{I_n^s(z_\mu)} = \sum_{\mu=0}^{m} \lambda_\mu \frac{I_n^s(z_\mu)}{I_n^s(z_\mu)} = 1,$$

where $b_\alpha = 1$ for $\alpha = 0$; $b_\alpha = A_\alpha$ for $\alpha = 1, \ldots, s$; and $b_\alpha = a_\alpha$ for $\alpha = s + 1, \ldots, n$.

We now consider the polynomial

$$N(z) \equiv \sum_{\mu=0}^{m} \frac{\lambda_\mu z_\mu^{\alpha_0}}{I_n^s(z_\mu)} \frac{g(z)}{z - z_\mu}. \tag{13}$$

The coefficient of z^m is $\sum (\lambda_\mu z_\mu^{\alpha_0}/I_n^s(z_\mu)) \neq 0$ by definition of α_0.

Let $\beta_0 = \min \{\alpha_0, m\}$ and $\gamma_0 = \alpha_0 - \beta_0$. Obviously, we have $n - s \leq \beta_0 \leq \min \{n, 2(n - s)\}$ and $0 \leq \gamma_0 \leq s$. By direct computation, using equations (10), we can show that the successive derivatives $N^{(\beta)}(z)$ for $\beta = 0, 1, \ldots, \beta_0 - 1$ at the point $z = 0$ are all equal to zero. Hence the polynomial $N(z)$ is divisible by z^{β_0}, and we obtain

$$\frac{N(z)}{z^{\beta_0}} = \sum_{\mu=0}^{m} \frac{\lambda_\mu z_\mu^{\gamma_0}}{I_n^s(z_\mu)} \frac{g(z)}{z - z_\mu} \equiv M^*(z). \tag{14}$$

Now $M^*(z)$ is a polynomial of degree $m - \beta_0$ exactly. Equation (14) may be interpreted as a Lagrange interpolation representation for $M^*(z)$ at the points $z_0, z_1, \ldots, z_m$. If we now consider any factorization of $I_n^s(z)$ into a product of polynomials of respective degrees s and $n - s$, say, $I_n^s(z) = p_s(z)\, q_{n-s}(z)$, we can multiply $M^*(z)$ by $q_{n-s}(z)$ and obtain

$$M^*(z) q_{n-s}(z) = \sum_{\mu=0}^{m} \frac{\lambda_\mu z_\mu^{\gamma_0}}{p_s(z_\mu)} \frac{g(z)}{z - z_\mu} \equiv \Lambda^*(z),$$

and we have

$$I_n^s(z) = \frac{p_s(z)\, \Lambda^*(z)}{M^*(z)},$$

as required.

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